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The Journal of Biochemistry, Vol. 46, No. 7, 1959

COMPARATIVE BIOCHEMICAL STUDIES ON ALCOHOLIC FERMENTATION WITH SPECIAL REFERENCE TO THE FLOCCULATION PHENOMENA IN YEAST CELLS

I. INHIBITION OF YEAST FERMENTATION BY URANYL ION

By MASAYO KIJIMA

(From the Research Laboratories of Kirin Brewery Co., Ltd., Yokohama)

(Received for publication, March 1, 1958)

One of the important factors in characterizing yeasts used in the praxis of alcoholic fermentation industry is the liability of the cells to flocculation (*1*), a quality which has generally been supposed to be correlated with the properties of the cell surface. On the other hand, evidence has been accumulating to indicate that certain biochemical activities take place on the cell surface. The alleged effectiveness of uranyl ions in specifically inhibiting the surface-bound enzymic processes as suggested by Rothstein (*2*) seemed to the present author to provide a tentative method for distinguishing between different yeast strains according to the nature of their cell surface. In the present study kinetic investigations on the uranyl inhibition of alcoholic fermentation were performed using various strains of brewer's bottom yeast. On the basis of the experimental results obtained, a tentative scheme of the inhibitory process is proposed. The influences of some intrinsic as well as environmental factors were also studied. In the present paper, the results obtained will be briefly described as follows.

EXPERIMENTAL METHODS

Bottom brewer's yeast was innoculated in wort (100 mg./100 ml.) and grown for 7 days at 7° (or 3 days at 25°). The harvested cells were washed on the centrifuge with a 50 times volume of dilute sodium hydroxide solution ($N/250$), then with the same amount of distilled water. Alkali-washing was done for the purpose of clearing the cell surface from contaminating substances such as proteins, dextrine, melanoidine, etc. contained in the culture medium. The fermentation capacity was found to be unimpaired by this procedure in any appreciable measure. The cells were suspended in distilled water in a concentration of 0.7 per cent (g. wet cells/v) and stored at 0° until use. No particular operation such as starvation was carried out; stress was laid on investigating the cells in a state as intact as possible. Fermentation rate was determined by measuring the evolution of carbon dioxide in a Warburg manometer under aerobic as well as anaerobic condition with glucose, fructose or maltose as the substrate and with potassium phthalate solution as the buffer (pH 4.4 unless otherwise stated). The rate of carbon dioxide evolution was expressed by the amount of carbon dioxide (μl.) evolved in 10 minutes per 7 mg. yeast cells (wet weight), the mean value of readings during the period from 60 to 90 minutes after the addition of sugar being adopted. Varied amounts of uranyl acetate were added to the reaction mixture before the addition of sugar and the grade of inhibition was determined. In every case two parallel experiments were carried out.

RESULTS

Changes in Reaction Rate during Fermentation—The velocity of carbon dioxide evolution was found to be subject to a gradual change with time (Fig. 1). As will be seen from the figure, there was always a more or less marked period of induction, usually of about 50 to 60 minutes, before the maximal rate of fermentation was reached. The induction period was somewhat longer with lower sugar concentrations and in the presence of the inhibitor (UO_2). The maximal levels of fermentation rate were usually maintained during a reaction period of 60–90 minutes, although some noticeable drop in the reaction rate was observed under aerobic conditions, especially with higher concentrations of sugar. With deteriorated cell materials that has been stored for a longer period after harvesting, there was an immediate and continuous decrease in the fermentation rate from the beginning. In the following parts of this study, fresh cells were always used and the reaction rates were estimated from the readings taken during a period from 60 to 90 minutes after the addition of sugar.

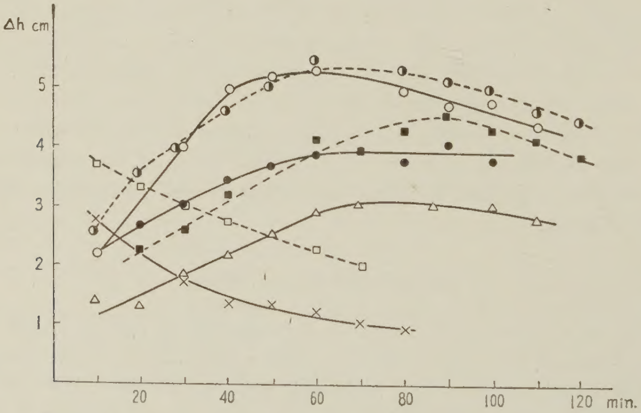

FIG. 1. Change in fermentation velocity during the lapse of reaction time.

Abscissa: Reaction time (minutes).

Ordinate: Rate of alcoholic fermenation as measured by carbon dioxide evolution (increase in manometric reading in 10 minutes).

○ glucose 0.56 M (aerobic); △ glucose 0.014 M (aerobic); ◑ glucose 0.28 M (aerobic); ■ glucose 0.28 M and UO_2^+ 7×10^{-5} M (aerobic); ● glucose 0.089 M (anaerobic); □ glucose 0.56 M (aerobic), aged yeast; × glucose 0.56 M (anaerobic), aged yeast; pH 4.4, temperature 30°.

Rate of Alcoholic Fermentation as Affected by Uranyl Ion—The addition of uranyl ions to the reaction mixture caused an inhibition of fermentation.

The inhibition was found to be essentially removed by washing the poisoned cells in the presence of polyphosphate (sodium hexametaphosphate, Koso Chemical Co.; 3 per cent aq.). On addition of uranyl acetate to the cells the inhibition equiliblium was readily attained, so that the same level of inhibition was obtained by measuring the fermentation rate immediately after the addition of the poison or long afterwards (*e.g.* 2 or 30 minutes). The relationship between the concentration of uranyl ion [I] and the reaction rate v_I in the presence of the poison was found to be in harmony with the following formula,

$$\frac{v_0-\alpha}{v_\mathrm{I}-\alpha}=1+k\,[\mathrm{I}] \qquad (1)$$

where v_0 is the reaction rate in the absence of uranyl ion and α and k are constants. The fitness of the formula will be best demonstrated by plotting the experimental results in terms of $((v_0-v_\mathrm{I})/[\mathrm{I}]$ *vs.* v_I, according to the following equation which is derived by rewriting the above equation.

$$\frac{v_0-v_\mathrm{I}}{k\,[\mathrm{I}]}=v_\mathrm{I}-\alpha \qquad (2)$$

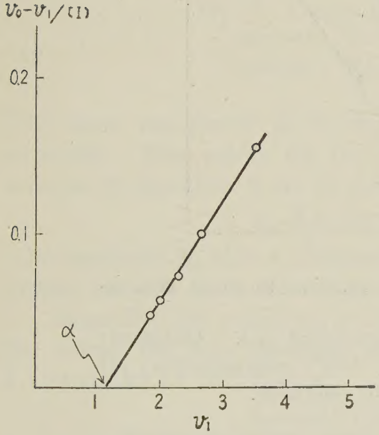

FIG. 2. Estimation of UO_2^{++}-uninhibited fraction of fermentation velocity.

Abscissa: Fermentation velocity inhibited (v_I). Ordinate: $(v_0-v_\mathrm{I})/[\mathrm{I}]$ where v_0 represents the velocity uninhibited, [I] being UO_2^{++} concentration.

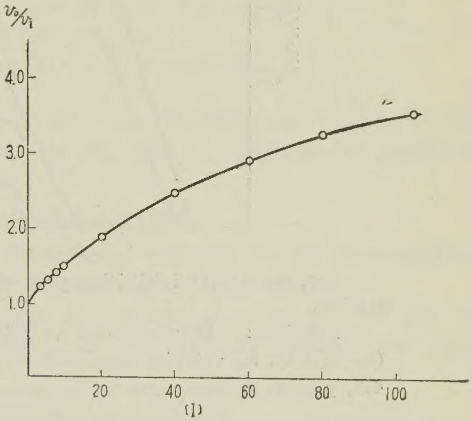

FIG. 3. Reaction pattern of inhibitor UO_2^{++} against fermentation velocity.

Abscissa: Concentration of uranyl ion, $(10^{-5}/6\,M)$ UO_2^{++}. Ordinate: Ratio of fermentation velocity, original to inhibited, pH 4.4, $v_0=6.60$ μl/10 min. Glucose concentration 0.25 M under aerobic condition.

In this way a straight line was obtained as will be seen in Fig. 2 (and also in Fig. 4). A plot of v_0/v_I *vs.* [I] as usual in the ordinary analysis of enzyme poisoning (3) gave rise to a curved line instead of a linear one which should be expected in the case of a genuine Michaelis-Menten type of inhibition (see Fig. 3).

The above results are best accounted for by assuming the fermentative activity of yeast cells to consist of two independent moieties, *i.e.*, an uranyl-sensitive and an uranyl-insensitive one. The magnitude of the latter part in the total fermentative activity is represented by α in the formula (Equation 1) which is solved graphically by measuring the intercept of the straight line with the abscissa in Fig. 2. The value of the constant k in the formula (Equation (2)) is obtained from the inclination of the straight line in the figure.

The effects of varied concentrations of the substrate on the uranyl inhibition was investigated. The validity of the previous formula was repeatedly confirmed as demonstrated in Fig. 4, from which the following values were computed for k and α.

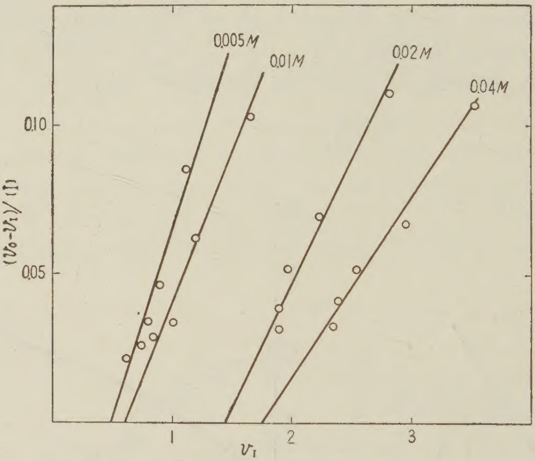

FIG. 4. UO_2^{++} inhibition pattern in relation to sugar concentration.

Abscissa: Fermentation velocity inhibited (v_I). Ordinate: $(v_0-v_I)/[I]$, v_0, velocity uninhibited; [I], UO_2^{++} concentration; pH 4.5, glucose fermentation under anaerobic condition.

Glucose concentration (M)	$k\times10^{-3}$ ($1/M$)	α	β ($=v_0-\alpha$)
0.04	36	1.76	3.90
0.02	50.5	1.45	3.40
0.01	60.1	0.62	3.06
0.005	66	0.50	2.39

First, the above set of data is plotted in Fig. 5 in terms of $1/k$ *vs.* [S]. A straight line was obtained in harmony with the following equation,

$$k=K_i/(1+K_{s\beta}[S]) \qquad (3)$$

This finding is indicative of the fact that the inhibition under investigation is of competitive nature with respect to the substrate, glucose in this case.

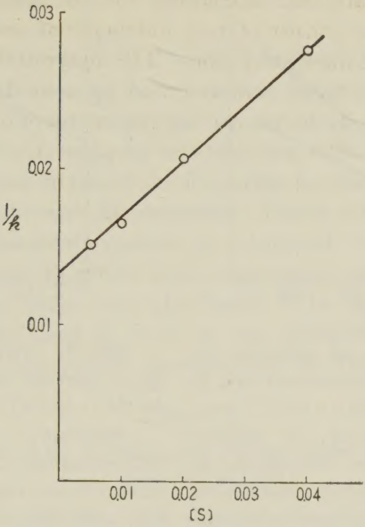

FIG. 5. Competitive nature of UO_2^{++} inhibition.
Abscissa: Glucose concentration.
Ordinate: Reciprocal of k in Equation (2).

The same was found to be the case also with fructose and maltose as the substrate. The values for the constants $K_{s\beta}$ and K_i obtained by graphical solution of Equation 3 are as follows;

$$K_i=8.3\times10^4\ (1/M),\quad K_{s\beta}=34\ (1/M)$$

The constants K_i and $K_{s\beta}$ represent the affinity constants of the fermentative system towards the inhibitor and the substrate (glucose), respectively.

Secondly, the relationship between the values of α (or β) and [S] in the above list was analyzed according to the ordinary formulation after Lineweaver and Burk;

$$k_{s\alpha}/\alpha=1+1/K_{s\alpha}[\mathrm{S}]\ ;\ k_{s\beta}/\beta=1+1/K_{s\beta}[\mathrm{S}] \qquad (4)$$

In the above equation β is introduced to denote the uranyl sensitive portion of fermentative activity; $\beta=v_0-\alpha$. Again a straight line was obtained (Figs. 6 and 7), from which the following values are obtained for the constants in the above equation.

$$K_{s\alpha}=30\ (1/M) \qquad K_{s\beta}=225\ (1/M)$$
$$k_{s\alpha}=3.3\ (\mu\mathrm{l.}/10\ \mathrm{min.}) \quad k_{s\beta}=4.8\ (\mu\mathrm{l.}/10\ \mathrm{min.})$$

The constants, $K_{s\alpha}$ and $k_{s\alpha}$, stand for the affinity constant of the uranyl insensitive moiety towards the substrate, and the velocity constant for this portion of the fermentative system, respectively. It ought to be noted in this connection that the values for the affinity constant of the sensitive moiety towards the substrate, $K_{s\beta}$, were computed, on the one hand, from Equation (4) (Fig. 7) and, on the other hand, from Equation (3) (Fig. 5) and are not in mutual accord. Indeed the former value was found to be six times as large

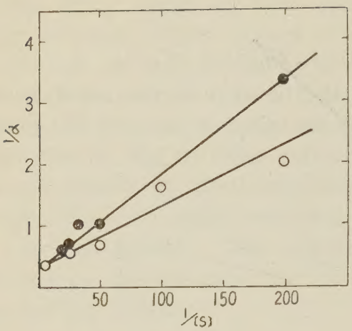

FIG. 6. The fitness of uranium insensitive portion of fermentation rate to the Michaelis and Menten's law.

Abscissa: Reciprocal of glucose concentration. Ordinate: Reciprocal of fermentation rate, uranium insensitive, pH 4.5; anaerobic, ○ potassium phthalate‘ ● sodium phthalate buffer solution.

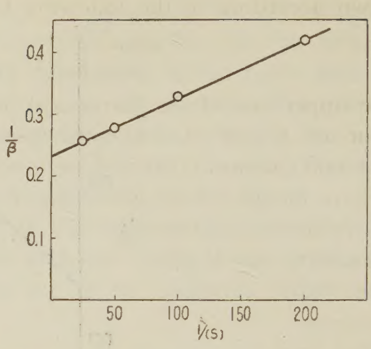

FIG. 7. The fitness of uranium sensitive portion of fermentation rate to the Michaelis and Menten's law.

Abscissa: Reciprocal of glucose concentration. Ordinate: Reciprocal of fermentation rate, uranium sensitive, pH 4.5; anaerobic.

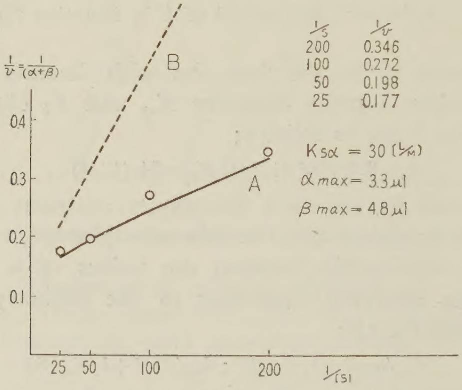

FIG. 8. Lineweaver-Burk for overall fermentation velocity.

Abscissa: Reciprocal of glucose concentration. Ordinate: Reciprocal of overall fermentation velocity; solid and broken curves were computed by the theory, using two different values for $K_{s\beta}$.

as the latter. The cause of this discrepancy has thus far been unexplained.

The above discovered circumstance that the fermentative system under investigation actually consists of two moieties each with a different affinity constant towards the substrate, should result in a failure of the ordinary Michaelis-Menten type of formulation in representing the velocity-substrate concentration relationship under investigation, which is fully shown from the curve given in Fig. 8 to be the case. The curves in the figure are drawn according to the following formula,

$$v_0=\alpha+\beta$$
$$=k_{s\alpha}\,K_{s\alpha}[S]/(1+K_{s\alpha}[S])+k_{s\beta}\,K_{s\beta}[S]/(1+K_{s\beta}[S]$$

The upper one of the theoretical curves in the figure was computed using a value of $K_{s\beta}$ of 34, while the lower one by assuming a value of 225, instead. The coincidence of the second curve with the experimental results speaks perhaps for the correctness of this $K_{s\beta}$ value (225) in the computation involved.

Influences of Intrinsic and Environmental Factors—The extent of uranyl-refractility of the fermenting system as estimated by the measure of the index $\alpha/(\alpha+\beta)$ was found to differ from one strain to another of the yeast organisms tested. The value was also found to be influenced by the nature of the fermenting sugar used as well as the conditions of the experiment. As will be seen from the results summarized in Table I, a value for the

TABLE I

Characteristics of the Inhibition with Different Sugars under Aerobic and Anaerobic Conditions

Sugar concentration 0.25 M; yeast cultured for 72 hours at 25° (100 mg./100 ml. wort).

		Glucose		Fructose		Maltose	
		Anaerob.	Aerob.	Anaerob.	Aerob.	Anaerob.	Aerob.
102	α (μl)	2.92	3.12	1.78	2.66	0.25	0.44
	β (μl)	2.64	2.56	3.65	2.95	2.03	2.54
	$\alpha/(\alpha+\beta)$(%)	52.5	54.9	32.8	47.4	11.0	14.8
	k*	31.2	26.4	14.4	19.2	129.6	124.8
201	α (μl)	1.25	1.80	0.65	0.85	0.07	0.33
	β (μl)	3.57	3.39	4.11	4.33	2.60	4.04
	$\alpha/(\alpha+\beta)$(%)	25.9	34.7	13.7	16.4	2.6	7.6
	k*	26.4	26.4	12.0	14·4	120.0	55.2
301	α (μl)	0.82**				0.25	0.30
	β (μl)	5.45**				4.65	4.60
	$\alpha/(\alpha+\beta)$(%)	13.1				5.1	6.5
	k*	37.2				3.5	2.0

* Inhibition constant, k at a substrate concentration of 0.25 M (see Equation (2)), a factor of 10^3 has to be multiplied to.

** Although at $[S]=0.25$ α could not be evaluated because of plots being deviated from Equation (2), at lower sugar concentration, it was succeeded and the values at $[S]=0.04$ is cited.

refractility-index as large as 50 per cent was obtained with Strain *102* (with glucose as substrate), while it was small in the case of Strain *301*. Among the three sugars thoroughly investigated (glucose, fructose and maltose) the first named was the most favorable substrate to support the uranyl-refractility. With maltose as the substrate on the contrary, the sensitivity towards uranyl was most enhanced. In some cases aerobiosis seemed to favor the resistance of the system against uranyl ions.

Another constant k in the Table, which represents the inhibition constant k at a substrate concentration of 0.25 M as used in this set of experiments, is determined by the nature of the sugar involved. This finding will be readily understood when we remember that the quantity k is a function of the affinity constant K_s of the fermenting system towards each sugar as opposed to the affinity constant towards the poison;

$$k=K_i/(1+K_s[S])$$

In fact, the values for k with maltose, glucose and fructose as the respective substrates were found to be in a ratio of 5 : 1 : 0.5 irrespective, of the yeast strain used (Table I). Aerobic condition of the experiments instead of anaerobiosis as was ordinarily the case in this study, was usually found to alter the results but little in this respect (Table I).

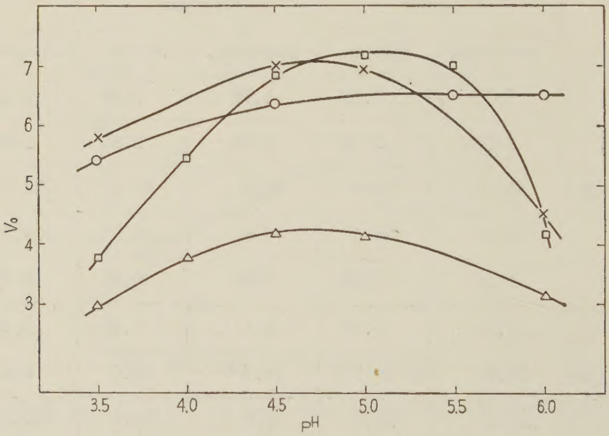

FIG. 9. The fermentation velocity in relation to pH.
Abscissa : pH. Ordinate : Velocity in μl/10 min., 7 mg. wet yeast, ○ glucose *102*, × glucose *301*, △ maltose *102*, □ maltose *301*.

With much higher substrate concentrations deviations from the above stated theory was encountered. No detailed investigations were attempted, but the limits of applicability with this respect are shown in Table II. The positive and the negative mark indicate the applicability and inapplicability of the formula (Equation (2)), respectively.

The effects of varied hydrogen ion concentrations has not been investigated in much detail. However, it will be noticed from the results shown in Fig. 9, that the uranyl-refractile fermentor, Strain *102*, with glucose as the substrate, show less prominent pH-dependency as compared with that in the cases of other more uranyl-sensitive systems.

TABLE II

The Limits of Fitness of the Theory (Formula 1) of Alcoholic Fermentation in the Presence of Uranyl Ion (UO_2^{++}) *in Terms of Various Experimental Conditions*

○ and × indicate, respectively fitness and non-fitness of the theory.

			102	*201*	*301*
Glucose	Anaerob.	0.25 *M*	○	××	××
		0.05 *M*	○	○	×
		0.04 *M*	○		○
		0.01 *M*	○		○
	Aerob.	0.25 *M*	○	×	×
Fructose	Anaerob.	0.25 *M*	○	×	×
		0.05 *M*	○	○	
	Aerob.	0.25 *M*	○	×	×
Maltose	Anaerob.	0.25 *M*	○	○	○
		0·05 *M*	○	○	○
	Aerob.	0.25 *M*	○	○	○

DISCUSSION

The most salient feature throughout the results of the present study is the fact that the fermenting activity of brewer's yeast consists of an uranyl-sensitive (β) and an uranyl-insensitive moieties (α), each contributing independently to the total rate of cellular fermentation. Through reaction-kinetic analysis of the experimental results, the values for the constants characterizing these two counterparts were computed to reveal that these two processes are significantly different from each other with respect to their apparent affinities towards the substrates, as well as the behaviour towards varied hydrogen ion concentrations. The relative weight of each moiety in the fermenting process was found to differ from one strain to another of the yeasts studied, although there seems to be some consistency in the relative order of magnitude of reaction-kinetic characteristics pertaining to the different substrates (see Table I). One point remaining open to later investigations is the discrepancy between the value of $K_{s\beta}$ derived from the analysis of the data in Fig. 5 (see Equations (2) and (3)) and that obtained from the plot of the calculated values of $1/\beta$ against 1/S (Fig. 7). Although there are no sufficient evidence for giving final decision between the two contradictory values of K_{s_β}, it will be noted in this connection that there is a certain possibility of the value for k having been overestimated in solving Equation 2 (Fig. 2). Actually, it was discovered in a later experiment (unpublished data) that there is a certain tendency of the plot in the said figure deviating from a straight line, especially when a sufficiently wide range of inhibitor concentration is concerned. A more detailed investigation is being carried out with this respect. As to the precise location of the uranyl-sensitive and -insensitive moieties of the fermentative activity of the yeast cells, no direct evidence has yet been achieved in the present study. The knowledge, however, of the inhibitory action of uranyl ions on various enzymes has led various authors to the common theory that this substance may be considered, at least tentatively, as an indicating reagent for a *surface enzyme* (*e.g.* Rothstein (*2*); yeast cells, Koshihara (*4*); Brain mitochondria. In such an elaborated system of metabolism as the alcoholic fermentation, it is hardly conceivable to assume that the cells should be equipped with two entirely different sets of machinery, one consisting of a series of uranyl-sensitive enzymes, and another of -insensitive ones. Taking into account the fact that the activities of the two moieties of the fermenting system in question are reaction-kinetically independent from each other, the most plausible scheme to be assumed will be as follows;

Uranyl-sensitive site (β) ↘
ensuing series of fermentative enzmes
Uranyl-insensitive site (α)↗

An additional condition for this explanation is that the rate of the overall fermentation reaction, in the present experiments, is limited by the initial step (α or β) which takes place at either of the uranyl-sensitive or -insensitive site of cell surface. This seems to be not in disaccord with the findings of previous authors that the uptake of some metabolites at the cell surface is often highly affected by uranyl ions (Rothstein (*2*)). An alternative explanation for the finding of the present study, on the other hand, will be that the test materials in each experiement consisted of, roughly speaking, two kinds of cells, one uranylsensitive and another -insensitive. The author is inclined to add a further assumption that the younger and the older cells represent, respectively, the sensitive and insensitive counterparts β and α. In fact, it was discovered with some yeast strains (*e.g.* Strain *102*) that the β-activity decreased with the lapse of culture period, while the α-counterpart remained almost undiminished. At any rate, it will be natural to assume that some differences in chemical structure at the cell surface make the material basis for the different behaviour of the α and β sites towards uranyl ion. Comparative studies of the chemical composition of the cell surface are being carried out with the strains used in the above experiemtnts. It will only be noted here that with two strains of *essentially* uranyl-sensitive strains (*301* and *303*) almost devoid of α-moiety, a significantly lower level of lipid was observed as compared with the strains (*101* and *102*) equipped with both α and β moieties of fermenting system. It will also be mentioned that washing with dilute NaOH, dilute HCl or with dilute ether-water mixture affected the uranyl-sensitive moiety more strongly than its counterpart.

SUMMARY

1. A comparative investigation was undertaken on the alcoholic fermentation of various strains of brewer's bottom yeast, with special reference to the inhibition caused by uranyl ions. The data obtained were analyzed from the reaction-kinetic point of view.

2. It was discovered that the fermentative system of the yeast cells consisted of two independently working moieties, one uranyl-sensitive (β) and another -insensitive (α). Some strains were discovered to be *essentially* uranyl-sensitive, being almost devoid of the α-moiety of fermenting activity, while others were found to be equipped with both α and β.

3. Reaction kinetic constants characterizing the two moieties of fermenting activity were computed through the analysis of the data obtained.

4. The results obtained were discussed in connection with the findings of previous investigators on the inhibition of enzyme reactions by uranyl ions.

The author wishes to express his thanks to the Directors of Kirin Brewery Co. Ltd. who has permitted the publication of experimental data in this report and to Prof. Emeritus Y. Okuda of Kyushu University and Dr. Y. Umeda and Dr. S. Kudo of the Laboratories of Kirin Brewery Co., Ltd. for their kind encouragement throughout the work and to Prof. A. Takamiya of Tokyo University for his kind discussions and advices in preparing this manuscript.

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The Journal of Biochemistry, Vol. 46, No. 7, 1959

CATABOLISM OF LECITHIN AND LYSOLECITHIN BY AVIAN TUBERCLE BACILLI

By MINORU HOSHINO

With the technical assistance of

HIROSHI SUZUKI

(*From the Department of Hygiene, Fukushima Medical College, Fukushima*)

(Received for publication, October 8, 1958)

Oxidation or hydrolysis of phospholipids by the enzymes derived from animal, plant, and microbiol sources have been studied by numbers of investigators. These works were reviewed by Zeller in 1951 (*1*) and by Deuel in 1955 (*2*). However, relatively little work has been done on the metabolism of the phospholipids in acid-fast bacteria, nevertheless the importance of the role of the lipids in the physiology of the bacteria (*3*).

Toda (*4*. *5*) and Toda and Urabe (*6*) reported that the human, bovine, frog, and avian types of tubercle bacilli obtained from the stock cultures, had lecithinase activity. They measured the activity by the titration method, that is, only the acids produced were titrated with alkali but the other degradation products were not analysed. The lecithin used was not highly purified and probably was contaminated with other phospholipids. Thus the lecithinase activity measured might have not represented the true activity.

It is noteworthy that the α-toxin of *Clostridium welchii* is lecithinase itself (*7*). The α-toxin is the first exotoxin demonstrated to owe its activity to an enzyme. It decomposes lecithin into phosphorylcholine and diglyceride and is inhibited by the antitoxic sera of *Clostridium welchii* type A.

Studies have been made in our laboratory on the phospholipase B of the avian tubercle bacilli (*8–10*), and the properties of the partially purified enzyme have been reported. The activity of the phospholipase B was measured by the decrease of the hemolytic action caused by the degradation of lysolecithin. The mechanism of the hemolytic action, however, is a disputable problem, and it is questionable whether the decrease of the hemolytic action of the reaction system corresponds to the progress of the hydrolysis of lysolecithin.

It has been pointed out by many investigators that, in the biological system, there is no enzyme without corresponding endogeneous substrate (*11–13*). It is known that no choline phospholipid is found in the cells of tubercle bacilli. Thus our findings of phospholipase B activity in these bacilli is not compatible with the theory mentioned above. In this connection, attempts were made to investigate whether lysolecithin is actually catabolized by the tubercle bacilli.

This present paper is a detailed report on these investigations.

MATERIALS AND METHODS

Substrates—Dipalmitoleyl-L-α-lecithin (yeast lecithin), glycerylphosphorylcholine (GPC), and phosphorylcholine (PC) were kindly supplied by Dr. D. J. Hanahan of the University of Washington, U.S.A., Synthetic dimyristoyl-L-α-lecithin was obtained through the courtesy of Dr. E. Baer of the University of Toronto, Canada. Egg lecithin was prepared according to Pangborn's method *via* cadmium chloride double salt (*14*). Lysolecithin was extracted from rice and purified by Iwata's method (15).

Preparation of Bacterial Suspension—Avian tubercle bacilli (strain Takeo) was kindly furnished by Dr. K. Matsui of the National Sanatorium Toneyama Hospital, Japan, and were grown on the modified Long's liquid synthetic media (sodium glutamate instead of asparagine) for 5 days, then harvested and washed well with $1/15\,M$ phosphate buffer (pH 7.0) using blendor, centrifuge, and finally Büchner's funnel. The washed bacteria were suspended $1/10\,M$ phosphate buffer (pH 7.0) to make the concentration of 20 mg. wet weight per ml.

Methods—Oxygen uptake was measured in Warburg vessels at 37° by the usual procedure. The test system contained 1.0 ml. of the bacterial suspension, 0.5 ml. of the substrate in water and 0.3 ml. of water with or without various reagents. The center well of the vessel contained 0.2 ml. of KOH and a small strip of filter paper. The gaseous phase was air. Unless otherwise mentioned, this condition was used throughout the experiments.

Lecithin and lysolecithin in the resulting mixtures were determined by circular chromatography on silica treated paper (*16*). The reaction system contained 5 μM of lecithin or lysolecithin, 5 ml. of bacterial suspension and water to make a final volume of 10 ml. The method of extraction of the sample for chromatography was as follows: 10 ml. of the sample was centrifuged at $1{,}400 \times g$ for 5 minutes, and washed twice with 5 ml. of $1/15\,M$ phosphate buffer (pH 7.0 in the case of lecithin, and pH 7.7 in the case of lysolecithin). The supernatant and washings were combined and filtered. The filtrate was shaken vigorously in a separatory funnel with 3 ml. of chloroform, and centrifuged at $1{,}800 \times g$ for 5 minutes. The water layer was extracted twice more with 3 ml. of chloroform. It was necessary in extracting lysolecithin that the pH of the filtrate should be adjusted to pH 7.7 before every shaking. The clear chloroform extracts were evaporated under reduced pressure, and the residue was dissolved in 0.1 ml. of chloroform. The sample (0.05 ml.) was spotted on the filter paper pretreated with silicate. Chloroform-methanol (1 : 1) mixture was used as the developing solvent. Lysolecithin was determined also by its hemolytic action as described in the previous paper (*8*).

Paper chromatography of α-keto acid in the resulting mixture was performed according to Cavalini *et al.* (*17*). The test system contained 65 μM of lecithin or 10 μM of lysolecithin, 10 ml. of bacterial suspension, and water to make the total volume of 18 ml. The developing solvent was *n*-butanol saturated with 3 per cent aqueous ammonia or butanol-ethanol-water (4 : 1 : 4).

RESULTS AND REMARKS

A) *Fate of Lecithin and Lysolecithin on Oxidation by Avian Tubercle Bacilli*

Influences of Lecithin, Lysolecithin and Related Compounds on the Oxygen Uptake *of Avian Tubercle Bacilli*—The net oxygen uptake of the system increased by the addition of yeast lecithin, egg lecithin and synthetic dimyristoyl lecithin, in increasing order of effectiveness (Fig. 1). The difference in the degree of increase of net oxygen uptake by the lecithins could be attributed to the difference in the fatty acid components as these lecithins differ one another only in their fatty acids attached to GPC. The influence of the yeast lecithin on the net oxygen uptake increased with an increase in concentration of this lipid up to 20 μM.

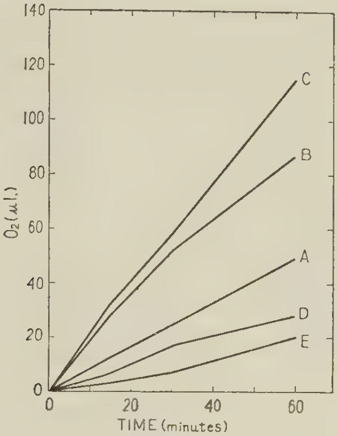

FIG. 1. Oxidation of lecithin.

A: Yeast lecithin, 1 μM D: Egg lecithin, 1 μM
B: Yeast lecithin, 10 μM E: Synthetic dimyristoyl lecithin,
C: Yeast lecithin, 20 μM 1 μM.

The effect of graded concentrations of lysolecithin on oxygen uptake is illustrated in Fig. 2. What differs from the case of lecithins is that the lowest level of the concentration tested, 1 μM, caused a marked increase in oxygen uptake, and that the oxygen uptake by higher levels of lysolecithin, 10 μM and 20 μM, caused smaller increases of oxygen uptake than in lecithin. This may be due to the complication of the effects of reaction products: *e.g.*, high concentration of oleic acid, which may be released from lysolecithin, is known to decrease the endogeneous oxygen uptake of tubercle bacilli (*20*), whereas low concentration stimulates the oxygen uptake as will be shown in the following paragraph, and the increase of oxygen uptake also has been observed with the increase of the concentration of glycerol, one of the possible degradation products of the lipid. This question requires further investigation.

The hemolytic action of the system disappeared near 30 minutes while the net oxygen uptake still followed a linear rate. Consequently, it was suggested that the lysolecithin was decomposed in the course of 30 minutes incubation, and that the oxygen uptake after that might have been caused by the degradation products of lysolecithin.

Glycerolmonostearate and β-glycerophosphate (β-GP), in the concentration of

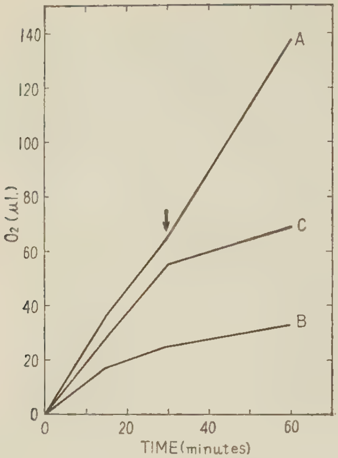

FIG. 2. Oxidation of lysolecithin.
A: Lysolecithin, 1 μM
B: Lysoleci-thin, 10 μM
C: Lysolecithin, 20 μM.
The arrow marks the disappearance of hemolytic action.

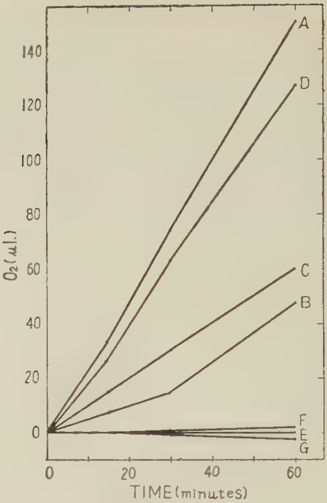

FIG. 3. Oxidation of glycerol, β-glycerophosphate, glycerolmonostearate, lysolecithin, choline, phosphorylcholine and glcerylphosphorylcholine.
A: Glycerol, 1 μM; B: β-Glycerophosphate, 10 μM; C: Glycerolmonostearate, 10 μM; D: Lysolecithin, 1 μM; E: Choline, 10 μM; F: Phosphorylcholine, 10 μM; G: Glycerylphosphorylcholine, 10 μM.

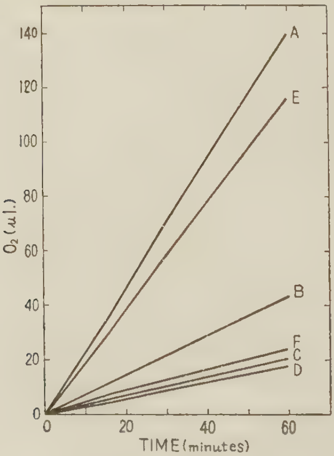

FIG. 4. Oxidation of fatty acids.

A: Oleic acid, 1 μM	D: Stearic acid, 1 μM
B: Linoleic acid, 1 μM	E: Lysolecithin, 1 μM
C: Palmitic acid, 1 μM	F: Lecithin, 1 μM.

1 μM did not increase the oxygen uptake. With 10 μM of these compounds the oxygen uptake was increased only in lower degree than with 1 μM of lysolecithin. Provided that glycerolmonostearate or β-GP was hydrolysed, glycerol would have been produced, then the glycerolmonophosphate or β-GP would have caused higher oxygen uptake than did lysolecithin if the stearate or phosphate produced simultaneously had no effects on the oxygen uptake of the system. GPC, PC and choline had only a slight effect on the oxygen uptake of bacterial suspension (Fig. 3).

The fatty acid components of egg lecithin are known to be mainly oleic, linoleic, palmitic, and stearic acids (*2*). The oxygen uptake of these fatty acids was studied and the time-course is shown in Fig. 4. Only oleic acid caused a higher oxygen uptake than that by lysolecithin. The effect of linoleic acid was intermediate. The increase of the oxygen uptake by palmitic and stearic acids were less than that by egg lecithin.

The increase of oxygen uptake caused by lecithin and lysolecithin were studied further. The test system contained 1 μM of the substrate in one side arm, another 1 μM in the other, 1 ml. of bacterial suspension in the main vessel, 0.2 ml. of 20 per cent KOH in the center well, and water to make a total volume of 2.5 ml. After equilibration, one side arm was tipped and the readings taken every 60 minutes, and then the other side arm tipped after 5 or 10 hours incubation.

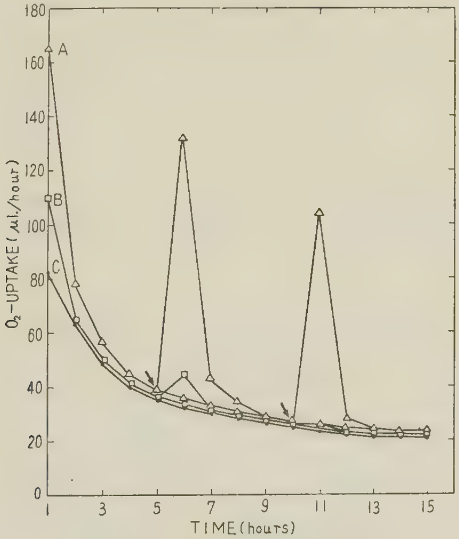

FIG. 5. Oxidation of lecithin and lysolecithin.
A: Lysolecithin, B: Lecithin, C: Endogeneous O_2 uptake
The arrow indicates the addition of lecithin or lysolecithin.

The rate of oxygen uptake was plotted for every one hour (Fig. 5). By the addition of lysolecithin at zero, 5 or 10 hours, the rate of oxygen uptake was immediately increased each time almost to the similar extent. The additions of lecithin at intervals of 5 hours also caused immediate increase in the rate of oxygen uptake. However, the effect of the addition of this lipid diminished stepwise from the first to the last addition. It seems that lysolecithin was oxidized in the system. Yanagisawa *et al.* (*18*) suggested that the non-ionic detergent, Triton W-R-1339, has some physicochemical action on the surface of the tubercle bacilli which have non-polar surface on the cell body. If the increase of oxygen uptake caused by the addition of lecithin was due only to the alteration of cell surface, detergent might also cause an increase of oxygen uptake, but Triton A-20 (a non-ionic detergent) did not cause any increase of oxygen uptake.

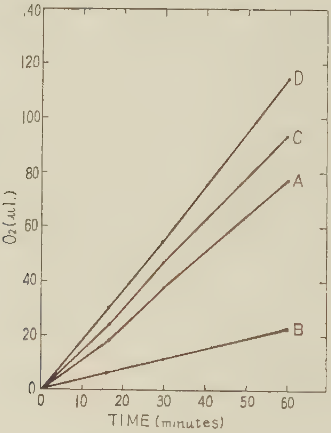

FIG. 6. Oxidation of partially hydrolyzed lecithin and lysolecithin.

A : Hydrolyzed lecithin, 1 μM ; B : Untreated lecithin, 1 μM ; C : Hydrolyzed lysolecithin, 1 μM ; D : Untreated lecithin, 1 μM.

Influences of Partial Hydrolysis of Lecithin and Lysolecithin—Acid hydrolysates of lecithin and lysolecithin were prepared according to Baer and Kates (*19*). As shown in Fig. 6, a higher net oxygen uptake was observed with hydrolyzed lecithin than with the untreated, while a lower net oxygen uptake with hydrolyzed lysolecithin than with the untreated. Bergström *et al.* (*20*) reported that certain unsaturated fatty acids, namely, oleic, linoleic, and linolenic acids caused the decrease of oxygen uptake of tubercle bacilli. Thus various effects of lecithin and lysolectithin on the oxygen uptake are probably attributed to the varieties of the fatty acids and other degradation products of the lipids in the resulting mixture.

Paper Chromatography of the Reaction Products—Paper chromatography showed that lecithin in the reaction mixture remained unchanged after 24 hours. Lysolecithin, on the other hand, disappeared from the mixture after incubation for 30 minutes. This agreed with the result that the hemolytic action of the reaction mixture disappeared after 30 minutes incubation period. This also confirmed the adequacy of using the hemolytic action of lysolecithin, as in our previous works, in measuring the phospholipase B activity of the bacilli. The α-keto acids in the reaction mixtures were also analysed by paper chromatography. Whichever the substrate was lecithin or lysolecithin, a clear spot of the R_f value similar to that of α-keto glutaric acid was detected on each chromatogram. The amount of the reaction mixture needed for the paper chromatography was about 10 times as much in the case of lecithin as in the case of lysolecithin. Samples of α-keto glutaric acid were added to the solutions obtained after the reaction with lecithin or lysolecithin, and the mixtures were chromatographed. However only single spot was observed near the R_f value corresponds to α-keto acid. According to the advice given by Prof. Uemura of Tohoku University, the chromatograms were developed again with the same solvent system, and two spots were clearly separated. Evidently one spot was not the α-keto glutaric acid but another compound. This agreed with the result that citrate and malonate failed to affect the oxidation of lysolecithin and the catabolic pathway of lysolecithin is not via TCA cycle.

Paper chromatography of fatty acids in the resulting mixture was performed according to Inouye and Noda's method (*26*). Several spots were detected, but they were not so clear as to be identified with known fatty acids.

B) *Enzyme System Involved in the Oxidation of Lysolecithin*

Effects of Various Reagents on the Oxidation of Lysolecithin—Additions of citrate, malonate, calcium, and fluoride to the oxidation system of lysolecithin had no

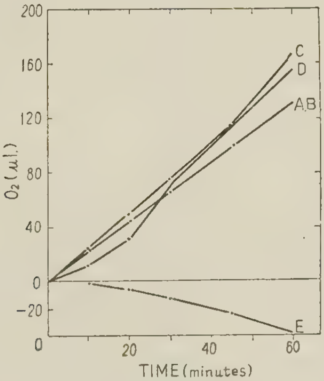

FIG. 7. Effects of various substances on the oxidation of lysolecithin.

A: Lysolecithin only, B: Lysolecithin plus malonate, citrate, cytochrome c, As, Ca, or F, C: Lysolecithin plus Mg^{++}, D: Lysolecithin plus DNP, E: DNP only, without lysolecithin.

effect on the oxygen uptake, while magnesium and 2,4-dinitrophenol activated it to some extent (Fig. 7). The fact that citrate and malonate had no effect may indicate that the catabolic path of lysolecithin is not via TCA cycle.

When 2,4-dinitrophenol was added in the absence of lysolecithin, the oxygen uptake observed was lower than the endogenous respiration, but in the presence of lysolecithin, net oxygen uptake was somewhat higher than that by lysolecithin only. Additions of cytochrome c (2.5×10^{-4} *M*) and arsenite (10^{-3} *M*) had no effect on the oxidation of lysolecithin.

The effects of several reagents which take part in flavin enzyme system were investigated. Flavin adenine dinucleotide (FAD) in the concentration of 5×10^{-4} *M* slightly increased the oxygen uptake, but in 10^{-3} *M* inhibited the oxidation of lysolecithin. Thus no marked increase in the addition of FAD was observed. However, some flavin enzymes are not activated by the addition of FAD. For instance, the activity of lactic oxidase of avian tubercle bacilli, is not increased by the addition of FAD (*21*). Acriflavin, which has a inhibitory action for the DPNH reductase (*22*), a flavin enzyme, inhibited the oxidation to a great extent. The addition of quinine, which is an inhibitor of esterase (*23*), and of FAD (*24*) caused decrease of oxygen uptake. It has been known that many flavin enzyme were strongly inhibited by Ag^+ or Cu^{++}. From these facts, it was supposed that flavin enzyme system are concerned with the catabolism of lysolecithin (Table I).

TABLE I

Effects of Reagents Participate in Flavin Enzyme System on the Oxidation of Lysolecithin

Reagent	Concentration (*M*)	Inhibition (%)
FAD	10^{-3}	25
	5×10^{-5}	(−15)
Acriflavin	10^{-3}	100
	10^{-4}	65
	10^{-5}	17
Quinine	10^{-3}	50
	10^{-4}	5
Ag^+	10^{-3}	100
	10^{-4}	80
	10^{-6}	14
Cu^{++}	10^{-2}	100
	10^{-3}	35

The effect of several SH reagents on the oxidation of lysolecithin were examined. *p*-Chloromercuribenzoate (PCMB), monoiodoacetate, Hg^{++}, and pyruvate (*25*) inhibited the oxidation of lysolecithin. Fig. 8 illustrates the inhibitory effects of PCMB in various concentrations. The effect of ferricyanide was very small compared with that of other SH reagents (Table II). Thus the participation of SH groups in the oxidation of lysolecithin is probable. If SH groups participate in this reaction, the activity inhibited by PCMB could be recovered by the addition of cysteine or glutathione.

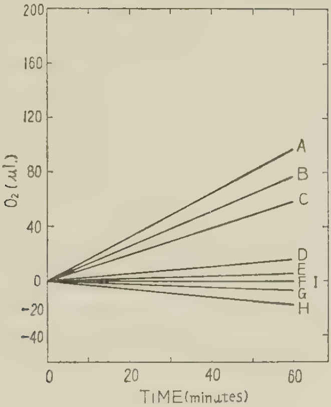

FIG. 8. Effects of PCMB in various concentrations on the oxidation of lysolecithin.

A: Lysolecithin only, B: 10^{-5} *M*, C: 2×10^{-5} *M*, D: 5×10^{-5} *M*, E: 7×10^{-4} *M*, F: 10^{-4} *M*, G: 7×10^{-4} *M*, H: 10^{-3} *M*, I: Endogenous O_2 uptake.

TABLE II

Inhibition of the Oxidation of Lysolecithin by SH Reagents

Reagent	Concentration (*M*)	Inhibition (%)
PCMB	10^{-3}	100
	10^{-4}	90
	10^{-5}	26
JAA	10^{-2}	92
	10^{-3}	46
	10^{-4}	19
Ferricyanide	10^{-3}	6
Pyruvate	10^{-2}	53
	10^{-3}	30
Hg^{++}	10^{-3}	100
	10^{-4}	90

Experiments were made, therefore, to observe the effect of cysteine. Since cysteine itself was found to be oxidized to a great extent by the bacilli, the concentration of PCMB added was so adjusted as to inhibit the oxidation of cysteine as well. The addition of cysteine to the system previously inhibited by PCMB, however, failed to recover the oxidative activity of the system. As attempt was made to inhibit the cysteine oxidase activity by the use of inhibitors such as NaN_3 or KCN. However, these substances, even in the concentrations insufficient to inhibit cysteine oxidase, already inhibited the oxidation of lysolecithin. Therefore the recovery effect of cysteine was not confirmed.

Effects of metal-chelating reagents on the oxidation of lysolecithin were studied. The reaction mixture was incubated, prior to the activity measurements, with metal-chelating reagents for 30 minutes at 37°. Ethylenediaminetetraacetate (EDTA) in the concentration of 10^{-2} M inhibited the oxidation by 10 per cent, while 10^{-3} M of 8-oxyquinoline, an Mg^{++}-chelating reagent, showed 25 per cent inhibition (Table III). The last result with the fact that Mg^{++} had an activating effect on the oxidation of lysolecithin. Only a slight effect was observed with diethyldithiocarbamate which chelates with Cu^{++}, although the addition of Cu^{++}, as described in the previous paragraph, reduced the oxidation of lysolecithin. Inhibitions by 10^{-3} M of NH_2OH, α, α'-dipyridyl, and *o*-phenanthroline were of 67 per cent, 55 per cent and 29 per cent respectively. An activation effect was observed with 10^{-3} M of nitroso-R-salt.

TABLE III

Effect of Metal-chelating Agents and Addition of Corresponding Metal Ions on the Oxidation of Lysolecithin

Reagent	Reagent concentration (M)	Inhibition (%)	Metal ion added	Inhibition (%)
EDTA	10^{-2}	10		
8-Oxyquinoline	10^{-3}	25	Mg^{++}	16
			Al^{+++}	4
			Zn^{++}	7
NH_2OH	10^{-3}	67	I^-	42
			Al^{+++}	16
			Zn^{++}	15
α, α'-Dipyridyl	10^{-3}	55	Fe^{++}	8
o-Phenanthroline	10^{-3}	29	Fe^{++}	9
Diethyldithiocarbamate	10^{-3}	5		
Nitroso-R-salt	10^{-3}	(+13)	Co^{++}	(+4)
KCH	10^{-2}	81		
	10^{-3}	55		
	10^{-4}	5		
NaN_3	10^{-2}	69		
	10^{-3}	27		

The inhibitory actions of the metal-chelating reagents were canceled to some extent by the addition of corrsponding metal ions. For example, the degree of inhibition by $10^{-3} M$ of 8-oxyquinoline was reduced by the addition of Mg^{++}, Zn^{++}, and Al^{+++} down to 16 per cent, 7 per cent, and 4 per cent respectively. Table III summarizes the effects of metal ions on the system inhibited by various metal-chelating reagents.

DISCUSSION

The results described above indicate that lysolecithin is oxidized by cell suspension of avian tubercle bacilli, but proof is lacking that lysolecithin is the intermediate in the course of degradation of lecithin.

According to the personal communication* and the report (*27*) of Dawson, lecithin increased the oxygen uptake of sperm cells. However, with the increased oxygen uptake neither the liberation of free choline, GPC, or PC, nor the change of fatty acid ester bonds in the lecithin was observed. He suggested that the increased oxygen uptake was not due to the enzymatic hydrolysis of ester bonds in the lecithin.

With regard to the results described in this paper, he suggested that the increase of oxygen uptake of avian tubercle bacilli with the addition of lecithin was not caused by an actual catabolism but due to a non specific stimulating effect on the endogenous respiration. To some extent it might be true. However, the result of paper chromatography for keto acids suggests that similar reaction occurred with lecithin as withlysolec ithin though the extent was quite small in former case. As mentioned before, about ten times as much amount of the reaction mixture of lecithin was needed for the detection of the spot on chromatogram as needed for that of lysolecithin. Evidence is lacking, however, to prove whether the spot observed on the paper chromatogram corresponds to the reaction product derived from lysolecithin and lecithin, or to the product from endogenous substances.

Moreover, he suggested that the addition of lysolecithin resulted the higher increase of oxygen uptake than with lecithin was also due to the permeability effect of lysolecithin, as lysolecithin is much more soluble than is lecithin. However disappearance of lysolecitin in the incubation mixture was confirmed by measuring hemolytic action and by paper chromatography. The quantities of oxygen uptake caused by the additions of lysolecithin were almost the same in the first addition and the second one as shown in Fig. 5. If the increase of oxygen uptake were due to the stimulating effect on the respiration of avian tubercle bacilli, the endogenous respiratory substances would have been reduced by the first addition of lysolecithin, and the quantity of oxygen uptake by the second addition might be reduced. These results suggest that lecithin was oxidized to some extent and lysolecithin to a great extent.

The decrease of lecithin and the appearance of lysolecithin as intermediate in the course of lecithin degradation could not be detected by circular chromatography up to 24 hours incubation, though lecithin apparently caused an increase in oxygen uptake. Hayaishi and Kornberg (*28*) recently reported similar results using a strain of *Serratia plymuthicum*, and they suggested that these results are due to the excessive lysolecithinase (phospholipase B) activity of the lecithinase fraction. In our previous experiments, no phospholipase A activity was found in the avian tubercle bacilli by the method of hemolytic action. In our case, therefore, different from the Hayaishi and Kornberg's, the main catabolic path of lecithin might not be through lysolecithin.

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* Dawson, R.M.C., personal communication, June 21, 1957.

The increase of oxygen uptake observed after the disappearance of lysolecithin was appeared to be due mainly to the fatty acids released, as GPC, GP, PC, and choline, the possible intermediates of hydrolytic degradation of lysolecithin, failed to cause appreciable increase of oxygen uptake. However, palmitic acid, one of the component fatty acids of lysolecithin, did not cause much increase of oxygen uptake as did lysolecithin.

Oxidation of lysolecithin was not decreased by the addition of 2,4-dinitrophenol, and also not affected by cytochrome c nor arsenite. Therefore the cytochrome system might not participate in this system. FAD had no effect on the oxidation of lysolecithin, but the other reagents inhibitory for flavin enzymes, *i.e.* acriflavine, quinine, Ag^+ and Cu^{++}, were effective. Thus flavin enzymes might participate in the system. Most of the metal-chelating reagents inhibited the oxidation of lysolecithin. It was observed that the oxidative activity was restored cy the addition of metals corresponding to the chelating reagents. Therefore the participation of metal ions such as Fe^{++}, Al^{+++}, Zn^{++}, Mg^{++}, and Co^{++}, or I^- on the oxidation of lysolecithin was suggested.

SUMMARY

The catabolism of lecithin and lysolecithin has been studied using the washed cell of avian tubercle bacilli (strain Takeo).

The oxygen uptake of this bacilli was increased by egg lecithin, yeast lecithin, synthetic L-α-dimyristoyl lecithin and rice lysolecithin. In particular, lysolecithin caused the highest increase of oxygen uptake. Degradation of lysolecithin was confirmed by chromatography and from the loss of hemolytic action. The breakdown of lecithin was not confirmed.

Several component fatty acids of lecithin, namely, oleic, linoleic, palmitic, and stearic acids, caused the increase of oxygen uptake. On the other hand, glycerolmonostearate, GPC, β-GP, PC, and choline had relatively small effects on the oxygen uptake.

Paper chromatography for α-keto acids in the reaction mixtures of lecithin and of lysolecithin showed single clear spot in both cases.

Oxidation of lysolecithin was activated by Mg^{++} and 2,4-dinitrophenol. Malonate, citrate, cytochrome c, arsenite, Ca^{++} and F^- had no effect on the oxidation. Participation of TCA cycle or of cytochrome system was quite improbable. Effect of FAD on the oxidation of lysolecithin was relatively small but acriflavine, quinine, Ag^+, and Cu^{++} had marked effects. Thus participation of flavine enzyme was suggested. SH reagents, such as PCMB, monoiodoacetate, Hg^{++}, Cu^{++} and pyruvate, all inhibited the oxidation of

lysolecithin. The recovery of the oxidative activity by the addition of cysteine or glutathione could not be confirmed due to the formation of H_2S, which is inhibitory for the respiration of tubercle bacilli. Participation of SH groups in the oxidation was suggested. Effects of metal-chelating reagents, *i.e.*, EDTA, 8-oxyquinoline, NH_2OH, α, α'-dipyridyl, *o*-phenanthroline, and nitroso-R-salt, were examined, and participation of Fe^{++}, Al^{+++}, Zn^{++}, Mg^{++}, Cu^{++}, I^- and Co^{++} in the oxidation of lysolecithin was suggested*.

This work has been done under the direction of Prof. K. Hoshishima and the author wishes to express his thanks to him for the constant guidance in the course of this work.

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The Journal of Biochemistry, Vol. 46, No. 7, 1959

A PROTEOLYTIC ENZYME OF STREPTOMYCES GRISEUS

II. AN IMPROVED METHOD FOR PURIFICATION OF A PROTEASE OF STREPTOMYCES GRISEUS*

By MASAO NOMOTO and YOSHIKO NARAHASHI

(*From the Instituse of Physical and Chemical Research, Tokyo*)

(Received for publication, November 7, 1958)

As previously reported (*1*), a remarkable amount of a protease is produced by *Streptomyces griseus* in the usual culture broth, together with streptomycin. Since the existence of this enzyme had not been noticed up to that time, the enzyme has been entirely discarded during the usual purification process in streptomycin production. In the preceding paper (*1*), some attempts were made to get the protease as a by-product of streptomycin manufacturing, and a method for isolating this enzyme was reported.

The above method, however, seemed to be unsuitable for the industrial application. Since salting out procedure with ammonium sulfate was employed for separating the enzyme from streptomycin, a large quantity of ammonium sulfate was introduced into the resulting streptomycin fraction and the subsequent purification of this antibiotic became difficult. Therefore, a further improvement has been required for the commercial production of the protease.

It was pointed out earlier by Kawabe *et al.* (*2*) that Kaken C-1 (SP-7) resin**, a high crosslinked salicylic acid type cation exchange resin, had a large adsorption capacity for streptomycin. Recently, it was also found by the present authors that this resin did not adsorb any appreciable amount of protease, whereas the lower crosslinked same type resin, Kaken C-1 (SP-3), had a large adsorption capacity for this enzyme (*1*). Results of these investigations have lead to the expectation that, by resin column treatment making full use of such a "molecular sieve" effect of this resin series, the protease and streptomycin, should be conveniently isolated from the usual culture broth of *Streptomyces griseus.* By successive resin column treatment, as expected, the protease and streptomycin could be easily isolated and they were further purified by the subsequent procedures. Crystalline preparations of the protease were obtainable through these procedures.

This improved method seems to be profitable to the industrial production of this enzyme and, actually, is put in practice by the Kaken Chemical Co.,

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* Most of these data have been presented at the Annual Meeting of the Agricultural Chemical Society of Japan held at the University of Tokyo in March, 1956.

** The sign "SP" shows the mole-ratio (×10) of phenol/salicylic acid used for the synthesis of Kaken C-1 resin series. Thus, the larger the SP values, the higher the crosslinkage of the resulting resin.

Ltd., one of the manufacturers of streptomycin in Japan.

In this paper is presented the improved method for purification of the protease of *Streptomyces griseus* (Streptomyces protease G).

EXPERIMENTAL

Assay

Assay for Protease Activity and Nitrogen Concentration—These assays were carried out by the methods described in the preceding paper (*1*): Modified casein-Folin color method was used for assaying protease activity (*PU*), and micro-Kjeldahl method or Folin color method for assaying protein concentration (*PN*).

Assay for Streptomycin—Streptomycin was determined by the usual bioassay method. Streptomycin unit was represented by the sign *SU* in this paper, for convenience.

Materials

Enzyme—Throughout the present work, the usual culture broth of *Streptomyces griseus* was used. This broth was produced for the industrial production of streptomycin by the Kaken Chemical Co., Ltd. and supplied from them.

Resins—The adsorption capacity of Kaken C-1 resin for the protease seemed to be greatly affected by the degree of the crosslinking in the resin structure and became larger at a lower crosslinking. Therefore, in order to choose a suitable species of this resin series for the present purpose, a screening test was carried out. In this test were used Kaken C-1 resin species (SP-3), (SP-5) and (SP-7) which differ from one another merely in the degree of the crosslinking. Five g. of each resin (60-80 mesh at swelled H-form) were buffered at pH 6.8 with $M/15$ sodium phosphate buffer (pH 6.8) and put into a column, 12 mm. in diameter. Through this resin column, about 100 ml. of the enzyme solution (pH 6.8) containing about 2.05 *PU* of protease activity were passed at the rate of 0.5 ml. per minute. Adsorption capacity for protease was calculated as the difference in the total activity between the original enzyme solution and the effluent solution. As shown in Table I, the higher the crosslinking of the resin, the smaller the adsorption capacity for the protease.

TABLE I

Influences of the Degree of Crosslinking in Resin upon Adsorption Capacity for Protease

Species of Kaken C-1 resin series	Degree of crosslinking	Apparent adsorption capacity (*PU*/g. *of resin*)
Kaken C-1 (SP-3)	Low	3.21×10^{-1}
Kaken C-1 (SP-5)	Medium	$1.11\times$,,
Kaken C-1 (SP-7)	High	$0.42\times$,,

About 4.1×10^{-1} *PU* of the enzyme was used per g. of the resin.
Apparent adsorption capacity was calculated as follows:
PU adsorbed = *PU* of original solution − *PU* of effluent.

Since the adsorption capacity of this resin series for streptomycin is essentially alike, it appears that Kaken C-1 (SP-7) resin is the most suitable for selectively adsorbing streptomycin leaving the protease in the effluent, and Kaken C-1 (SP-3) is the best for collecting the enzyme on account of its large adsorption capacity for the enzyme.

Methods

Preparation of the Enzyme from the Culture Broth—The culture broth supplied from the factory was immediately centrifuged to remove the mycelia and other insoluble matters. About 700–800 ml. of dark-brownish supernatant was obtained from each 1,000 ml. of the original culture broth.

Since there still remained a considerable amount of turbid materials, the resulting supernatant was treated by calcium phosphate gel method for its clarification, as mentioned in the preceding paper (*1*). That is, to 9,000 ml. of the above supernatant, 300 ml. of 1 M Na_2HPO_4 solution and 300 ml. of 1 M $CaCl_2$ solution were added successively at pH 7.8. After standing for 6 hours below 10°., the precipitated calcium phosphate gel was removed off by centrifugation. Thus, about 10,200 ml. of yellowish supernatant (including the washings with water) was obtained showing about 90 per cent recovery of the protease and almost all of streptomycin.

Successive Resin Column Treatment—(*I*) *Treatment by the First Column of Kaken C-1* (*SP-7*) *Resin*: Kaken C-1 (SP-7) resin (40–60 mesh at swelled H-form) was well suspended in 2,000 ml. of $N/2$ sodium hydroxide at room temperature over-night and was converted to Na-form. About 450 ml. of this Na-form resin was transferred into a column (3.6× 42 cm.) and washed slowly by passing about 1,000 ml. of $N/2$ NaOH and then about 5,000 ml. of water.

Through this column, 10,200 ml. of the above enzyme solution containing 62 *PU* of protease and 214×10^5 *SU* of streptomycin was passed at the rate of 10–15 ml. per minute. Then the column was washed with about 200 ml. of water and the washings were combined with the effluent solution. In this effluent, there were contained about 90 per cent of protease and considerable amounts of the coloring matter but not streptomycin. Streptomycin which was almost completely adsorbed on the column was eluted by passing about 1,200 ml. of $N/2$ HCl at the rate of 10–15 ml. per minute. About 90 per cent of the adsorbed streptomycin were recovered in this eluate. Further purification of streptomycin is easily accomplished by the usual process.

(*II*) *Treatment by the Second Column of Kaken C-1* (*SP-3*) *Resin*: The above effluent passing through the first column was successively passed through the second column composed of Kaken C-1 (SP-3) for collecting the protease. The procedure adopted for this treatment was as described before (*1*). About 240 ml. of Kaken C-1 (SP-3) resin (60–80 mesh at swelled H-form) were previously buffered at pH 6.2 and put into a column (2.6×44 cm.). Through this column, 10,400 ml. of the above effluent was passed at the rate of 8–10 ml. per minute. The second column adsorbing the enzyme washed with about 1,200 ml. of water. In this effluent and washings, there were contained greater portion of coloring matter, proteinous impurities and a small amount of the enzyme. The enzyme almost completely adsorbed on the column was then eluted by passing about 2,900 ml. of Clark-Lub's $M/2$ sodium borate buffer (pH 9.2) containing $M/2$ KCl at the rate of 6–8 ml. per minute. The appearance of elution process is shown in Fig. 1.

The eluate was divided into 2 parts: Fraction I—about 900 ml. of enzyme-rich solution, and fraction II-about 2,000 ml. of the remains. In the fraction I, about 56 per cent of the adsorbed protease were recovered with ten-fold purity against the original broth.

Crystallization—Crystallization of the protease from the above fraction I was also

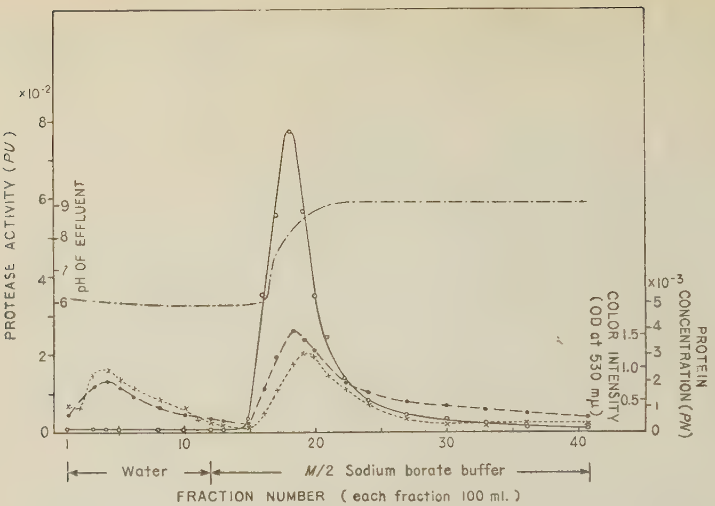


FIG. 1. Elution process for the protease adsorbed on Kaken C-1 (SP-3) resin column.

Resin column : 2.6×44 cm., 240 ml. of resin, pH 6.2.
Protease adsorbed : 50.1 *PU.*
Washing : 1,200 ml. of water, 8–10 ml./minute.
Elution : 2,900 ml. of *M*/2 sodium borate buffer (pH 9.2) containing *M*/2 KCl, 6–8 ml./minute.

—○— protease activity, —●— protein concentration,
--×-- color intensity, —·— pH.

carried out as reported before (*1*). The enzyme was precipitated by addition of 40 g. ammonium sulfate per 100 ml. and collected on a Buechner funnel with the aid of Hyflosupercel. After dissolving the enzyme in a small amount of 0.02 *M* calcium acetate solution, the enzyme solution was dialyzed over night against a large amount of 0.02 *M* calcium acetate solution at 4°. About 80 per cent of the protease contained in the fraction I were detected in the dialyzed solution.

From the above solution, the enzyme was precipitated again by addition of 3 volumes of acetone below 10° and collected by centrifugation. The precipitates resulted were dissolved in a minimum volume of 0.1 *M* calcium acetate solution, and a slight amount of insoluble matter, when it remained, was removed by centrifugation. In this concentrated enzyme solution were recovered about 80 per cent of the enzyme initially contained in the dialyzed solution. The purity of this preparation was twenty-fold higher than that of the original broth.

To this highly purified enzyme solution, a few drop of cold acetone was added until a very slight cloudiness scarcely appeared, and the mixture was kept in a tightly stoppered tube in a refrigerator for several days with occasional addition of 1–2 drops of cold acetone. Then, small needle crystals of the protease were gradually formed (Fig. 2). It was still hard, however, to prevent the contamination of amorphous protease into the crystalline preparation, as described before (*1*).

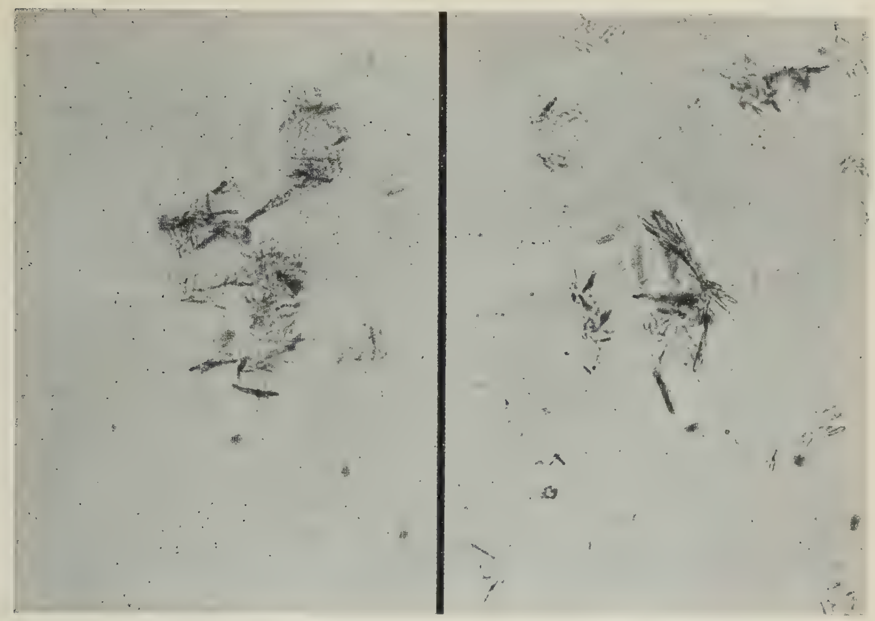


FIG. 2. Crystalline protease of *Streptomyces griseus* (×600).

RESULTS

Preparation of the Enzyme Solution from the Culture Broth—After removing the mycelia by centrifugation, the resulting supernatant was successfully clarified by the calcium phosphate gel method as described in the Methods. By this treatment, a yellowish clear solution was obtained. In this solution 80–90 per cent of the protease and about 90 per cent of streptomycin were detected. The suitable concentration of Na_2HPO_4 and $CaCl_2$ required for this treatment was found to be $M/30$–$M/40$.

On the other hand, when the culture broth was kept at 25-35° overnight, mycelia in the broth came up to the surface. After scraping the floating cakes of mycelia, the culture broth could be easily filtered by use of a Buechner funnel. Thus, about 700–800 ml. of yellowish filtrate was obtained from each 1,000 ml. of the broth. This filtrate is also available for the present purpose. In this case, however, a slight decrease in protease activity and an accumulation of histamine in the resulting filtrate occurred. Though a clear enzyme solution was obtainable more easily in this method than in the former method, the histamine accumulation brought about a difficulty in the purification of streptomycin.

Successive Resin Column Treatment—From the results of the screening test, it was proved that Kaken C-1 (SP-7) resin was useful for separating streptomycin from the protease and Kaken C-1 (SP-3) for collecting the enzyme. By using these resins successively, separation and purification of the above biological products were successfully accomplished as described in the Methods.

The orginal enzyme solution prepared from the culture broth was first passed through the column of Kaken C-1 (SP-7) and next passed through that of Kaken C-1 (SP-3), successively. Thus, the antibiotic was adsorbed on the first column and the enzyme on the second column. The adsorbed substances were eluted from the columns with diluted HCl solution and *M*/2 borate buffer, respectively.

Through this treatment, about 80 per cent of streptomycin, and about 30–40 per cent of the protease were recovered for the solution before charged.

The suitable amount of Kaken C-1 (SP-7), which adsorbes the whole streptomycin but not the protease, seems to be about 2 ml. (at swelled Na-form) per 10^5 *SU* of streptomycin, and that of Kaken C-1 (SP-3) about 5 ml. or less (at swelled H-form) per 1 *PU* of the protease.

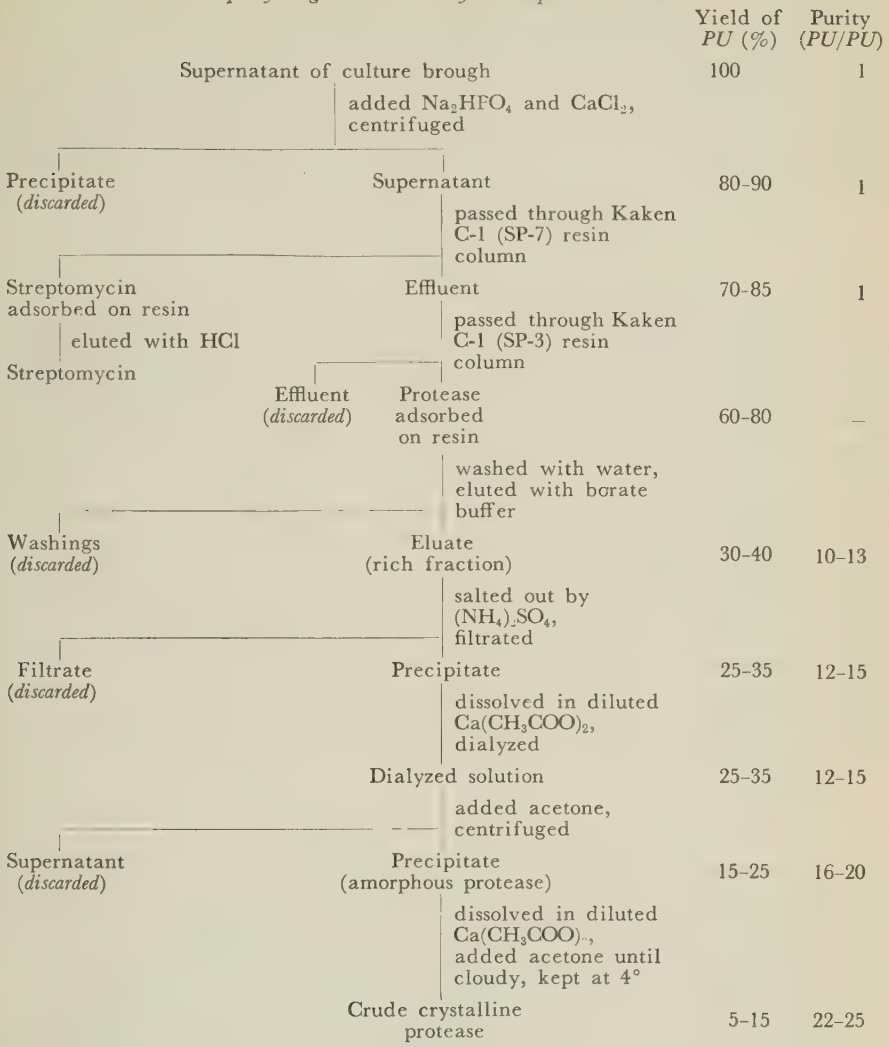
TABLE II

A Representative Result on the Purification of Streptomyces griseus Protease by the Improved Method

Fraction	Volume (*ml.*)	Protease			Purity (*PU*/*PN*)	Streptomycin		
		Conc. (*PU*/*ml.*)	Total (*PU*)	Yield (%)		Conc. (*SU*/*ml.*)	Total (*SU*)	Yield (%)
Supernatant of culture broth	9,000	$\times 10^{-3}$ 7.78	70.02	100	1.12	2,560	$\times 10^{-5}$ 230	100
Solution prepared by Ca-phosphate method	10,200	6.09	62.12	88.7	1.08	2,100	214	93.1
Effluent passed through Kaken C-1 (SP-7) resin column	10,400	5.49	57.10	81.5	1.32	77	8	3.5
Apparent amount of adsorbed enzyme	—	—	5.02	7.2	—	—	206	89.5
Eluate with HCl	1,200	—	—	—	—	15,400	185	80.3
Effluent passed through Kaken C-1 (SP-3) resin column	10,400	0.67	7.00	10.0	0·24		—	
Apparent amount of adsorbed enzyme	—	—	50·10	71.5	3 62	—	—	—
Washings with water	1,200	0.29	0.35	0.5	0.20	—	—	—
Eluate with borate buffer (I)	900	31.10	27.99	40.0	11.60	—	—	—
Eluate with borate buffer (II)	2,000	2.75	5.50	7.9	2.44	—	—	—
Dialyzed solution of salting out precipitate from fraction (I)	320	68.90	22.05	31.5	14.40	—	—	—
Solution of acetone precipitate	55	301.61	16.59	23.7	19.60	—	—	—
Solution of crude crystals	20	402.45	8.05	11.5	24.80	—	—	—

Scheme 1

Summary of Purification Procedures for Streptomyces griseus Protease by the Improved Method


Yield of PU (%)
Purity (PU/PU)
Supernatant of culture brough
100
1
added Na_2HPO_4 and $CaCl_2$, centrifuged
Precipitate (*discarded*)
Supernatant
80–90
1
passed through Kaken C-1 (SP-7) resin column
Streptomycin adsorbed on resin
eluted with HCl
Streptomycin
Effluent
70–85
1
passed through Kaken C-1 (SP-3) resin column
Effluent (*discarded*)
Protease adsorbed on resin
60–80
–
washed with water, eluted with borate buffer
Washings (*discarded*)
Eluate (rich fraction)
30–40
10–13
salted out by $(NH_4)_2SO_4$, filtrated
Filtrate (*discarded*)
Precipitate
25–35
12–15
dissolved in diluted $Ca(CH_3COO)_2$, dialyzed
Dialyzed solution
25–35
12–15
added acetone, centrifuged
Supernatant (*discarded*)
Precipitate (amorphous protease)
15–25
16–20
dissolved in diluted $Ca(CH_3COO)_2$, added acetone until cloudy, kept at 4°
Crude crystalline protease
5–15
22–25

Kaken C-2 (X-5), a high crosslinked phenoxyacetic acid type cation exchange resin, was also useful instead of Kaken C-1 (SP-7) for adsorbing streptomycin, as previously noted by Kawada *et al.* (*3*).

For adsorbing the enzyme, other kinds of resin such as Duolite C-10, Duolite S-30 or Kaken Phenol-type resin were also utilizable, though the adsorption capacities of these resins for the protease were smaller than that of Keken C-1 (SP-3).

Crystallization—Crystallization of the protease from the above enzyme-rich eluate was carried out as described before (*1*), and the similar results were also obtained. From the eluate, the enzyme was conveniently concentrated and purified by salting out and acetone precipitation, and finally crystallized from acetone solution. However, this crystalline preparation was still contaminated with small amounts of amorphous protease.

A representative results on the purification of the protease by the improved method are summarized in Table II. The whole purification procedures are also shown in Scheme 1 in which the mean value of the yield and purity of the protease in each step are noted together.

DISCUSSION

By the above improved method, a great defect of the previous purification method seems to be eliminated satisfactorily, and the protease and streptomycin, respectively, could be successfully isolated from the culture broth.

Moreover, this improved method has the three distinguish advantages; the simplicity in process, the easiness of application to the future expansion of production scale and the good yields of both protease and streptomycin. This method, therefore, has been practically brought into operation by the Kaken Chemical Co., Ltd.

A weak point still remained in the present method is a difficulty in removing mycelia. Since there are abundant amounts of mycelia in the usual culture broth, it is not convenient to remove them by usual centrifugation or filtration. Although the mycelia are easily removable by warming the broth for 24 hours, such a treatment is accompanied by the accumulation of histamine in the filtrate. Since the contamination of increased amounts of histamine in the filtrate makes the further purification of streptomycin complicated, some further devices are needed on this subject.

SUMMARY

A remarkable amount of a protease is produced by *Streptomyces griseus* in the usual culture broth, together with streptomycin. By successive resin column treatments based on an appropriate use of the "molecular sieve" effect of Kaken C-1 resin series, the protease and streptomycin could be easily isolated from the broth, and were highly purified by the subsequent procedures.

The filtrates of the broth were first passed through the column of Kaken C-1 (SP-7), a highly crosslinked cation exchange resin, and next passed through the column of Kaken C-1 (SP-3), a highly porous same type resin. Thus, streptomycin was adsorbed on the first column and the protease on the second one. Then, the enzyme adsorbed on the column was highly purified by the subsequent procedures such as displacement chromatography in elution process, salting out, and acetone precipitation. The protease was finally crystallized as needles from acetone solution.

On the other hand, streptomycin adsorbed on the first column was eluted with diluted mineral acid and successfully purified by the usual process.

This improved method seems to be suitable for the industrial production of the protease as a by-product of the streptomycin manufacturing.

The authors wish to express their thanks to Dr. T. Akahira, Dr. M. Yanagita, Prof. K. Sakaguchi, Prof. K. Okunuki and Dr. B. Hagihara for their kind guidance and useful suggestions. The authors are also grateful to Mr. S. Fujia for preparation of the enzyme and to Mr. H. Kawabe for resins, beside their helpful advices during the course of this work. Thanks are also due to the Kaken Chemical Co., Ltd. for the continual supply of culture broth.

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STUDIES ON THE BIOSYNTHESIS OF SILK FIBROIN

II. NON-UNIFORM LABELING OF SILK FIBROIN SYNTHESIZED IN VIVO

By KENSUKE SHIMURA, HIRONOBU KOBAYASHI,
REIKO HOSHI AND JIN SATO

(*From the Laboratory of Biochemistry, Department of Agricultural
Chemistry, Tohoku University, Sendai*)

(Received for publication, November 8, 1958)

It is generally assumed that the biosynthesis of protein takes place either by a mechanism in which the component amino acids combine simultaneously on a sequence-determining template or by a stepwise process. A useful method to distinguish the difference between the simultaneous and stepwise process is based on the comparison between the specific activities of amino acid isolated from different positions in the protein molecule. This method has been developed greatly by Anfinsen and his co-workers (*1–6*). A protein molecule synthesized by complete template mechanism will incorporate the labeled amino acid residues equally into a molecule of the protein (*9–15*). On the other hand non-uniform labeling of amino acid residues will support the conception of protein formation as a stepwise process (*1-8*).

In the present paper an attempt was made to elucidate the mechanism by which the silk-gland tissue synthesizes fibroin from the view point of labeling mode of amino acids residues in the fibroin molecule.

A preliminary report of these experiments has been published (*8*).

MATERIALS AND METHODS

Silkworms (*Bombyx mori*, L: a hybrid "Si 122 X Nichi 122") at the third to fifth day of the fifth instar were used.

Glycine-1-C^{14} and Alanine-1-C^{14} were obtained from the Radiochemical Centre, Amersham, England. Their specific radioactivities were 3.54 μc. and 4.2 μc. per mole respectively. The injection of the radioactive amino acids into silkworm was carried out as reported previously (*16*).

Preparation of Radioactive Silk Fibroin—Preparation of radioactive fibroin from the posterior silk-gland of silkworms, which were injected with the radioactive amino acids, was carried out by two methods, namely, modified Griffin's method and the trichloroacetic acid (TCA) method. Details of these fractionating procedures have also been presented previously (*16*).

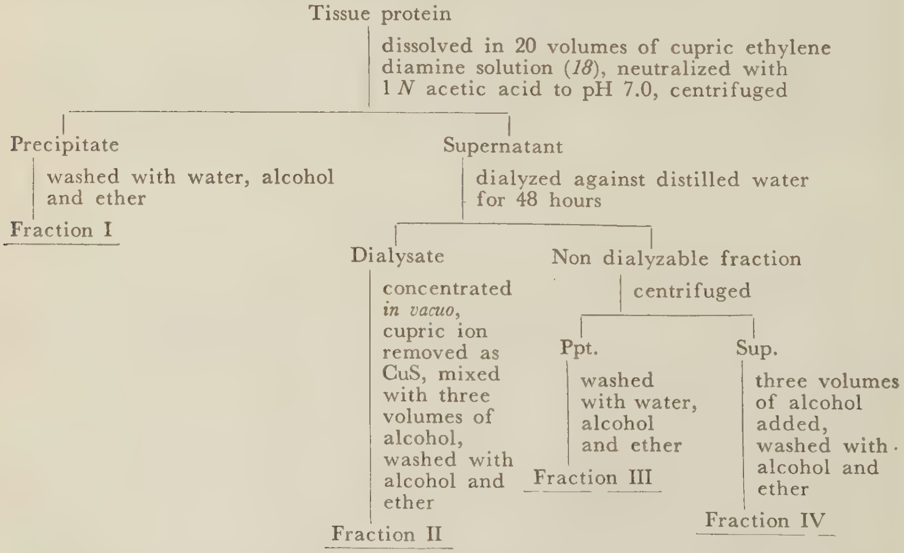
Isolation of N-terminal and non N-terminal Amino Acids—Preparative paper chromatography was used to isolate the N-terminal and non-N-terminal amino acids with the aid of the DNP-method (*17*). Radioactive fibroin and an equal weight of $NaHCO_3$ were suspended in 10 times the weight of water in a round bottomed flask of convenient size. To this was added twice the volume of an absolute ethanolic solution (5 per cent of 2,4-dinitrofluorobene (DNFB)), and the mixture was shaken for 4 hours at room temperature in a dark place. The insoluble DNP-fibroin was collected on filter paper and washed thoroughly with distilled water, ethanol, and ether to remove excess DNFB, dinitrophenol and free DNP-amino acids.

N-terminal DNP-Amino Acids—The DNP-fibroin was hydrolyzed with 6 *N* HCl in a sealed tube for 8 hours at 105°. The hydrolyzate was transferred to a separating funnel, diluted to 1 *N* HCl and the N-terminal DNP-amino acids were extracted with five portions of ether. The aqueous layer was reserved for preparation of non-N-terminal amino acids. The combined ether extract was evaporated in vacuo, and the residue was applied to a filter paper sheet (Toyo Roshi No. 50) in 30 cm. streak and developed in darkness by ascending technique with a solvent system of butanol-ammonia. A known mixture of standard DNP-amino acids, dinitrophenol and DNFB was run at each end of paper and used as a guide in determining the location of the desired DNP- amino acids.

The band of paper containing DNP-glycine or DNP-alanine was cut out, and eluted by permitting distilled water from a trough in a closed chamber to pass over the paper by capillary action. As this solvent system gave no satisfactory separation of DNP-glycine and DNP-serine, it was followed by a rerun of the eluate from the DNP-glycine containing band in a solvent of 0.1 *M* phosphate buffer (pH 7.0). The eluate of DNP-amino acid was evaporated to dryness, the residue was dissolved in acetone, and its optical density at 345 mμ was read on the Beckman Model DU spectrophotometer.

SCHEME 1

Fractionation of the Tissue Protein of Posterior Silkgland


Tissue protein
dissolved in 20 volumes of cupric ethylene diamine solution (*18*), neutralized with 1 *N* acetic acid to pH 7.0, centrifuged
Precipitate
washed with water, alcohol and ether
Fraction I
Supernatant
dialyzed against distilled water for 48 hours
Dialysate
concentrated *in vacuo*, cupric ion removed as CuS, mixed with three volumes of alcohol, washed with alcohol and ether
Fraction II
Non dialyzable fraction
centrifuged
Ppt.
washed with water, alcohol and ether
Fraction III
Sup.
three volumes of alcohol added, washed with alcohol and ether
Fraction IV

An aliquot of the acetone solution (containing 0.2 to 2.0 μM of DNP-amino acid) was transferred to a counting planchet of 25 mm. in diameter and dried up carefully under an infra-red lamp. The radioactivity was measured on a Geiger-Müller counter with a thin mica end window (1.43 mg./cm^2.). Within a range of 0.2 to 2.0 μM of radioactive DNP-amino acid, the specific radioactivity was found to increase almost linearly as the amount of DNP-amino acid increased, therefore, no correction for self adsorption was adopted. Care was, however, taken to mount nearly equal quantities of both DNP-amino acids derived from N-terminal and non-N-terminal amino acid residues on the counting planchet. Counting was continued for a period sufficient to insure that the statistical error was not more than 3 per cent.

Non-N-terminal DNP-Amino Acids—The acidic aqueous solution, which was kept in the procedure of extracting N-terminal DNP-amino acids, was treated with DNFB in the usual way, and non-N-terminal DNP-glycine and DNP-alanine were isolated as described above.

Fractionation of Tissue Protein—The tissue protein, which was obtained in the procedure of preparation of fibroin by the TCA method, was dissolved into cupric ethylenediamine solution according to the method of Coleman and Howitt (*19*). Details of the fractionation scheme are given in Scheme 1.

RESULTS

Typical results are given in Tables I, II and III, all of which show obviously non-uniform labeling of fibroin synthesized in the posterior silk-gland. N-terminal glycine of fibroin isolated by Griffin's method had, shortly after the injection of radioactive amino acid, a radioactivity considerably higher than that of non-N-terminal glycine. However, the activity of N-terminal glycine decreased with time and was about half of that of non-N-terminal glycine 20 to 40 minutes after injection. A similar, but more clear-cut result was obtained with fibroin prepared by the TCA method. The data are shown in Table III. The activity of N-terminal glycine was about five times as high as that of non-N-terminal glycine 30 minutes after the injection, but both radioactivities became nearly equal at one and a half hours, and finally at 18 hours the N-terminal glycine had no radioactivity in contrast to a considerably high activity of non-N-terminal glycine.

In the previous paper, we suggested a possibility of the occurrence of

TABLE I

Comparison of Radioactivity between N-terminal and non-N-terminal Glycine of Fibroin Prepared by Griffins's Method

Time after injection[1] (*minutes*)	Radioactivity of DNP-glycine		Ratio[2]	Radioactivity of fibroin (*c.p.m. per mg.*)
	N-terminal (*c.p.m. per μM*)	Non-N-terminal (*c.p.m. per μM*)		
5	50.8	31.8	1.60	50
10	40.9	56.3	0.73	70
20	35.2	65.2	0.54	120
40	28.4	71.0	0.40	141
60	23.8	68.4	0.35	151

1) Each silkworm was injected with 2×10^4 c.p.m. of glycine-1-C^{14}.

2) c.p.m. of N-terminal/c.p.m. of non-N-terminal.

TABLE II

Comparison of Radioactivity between N-terminal and Non-N-terminal Alanine of Fibroin Prepared by Griffin's Method

Time after injection[1] (*minutes*)	Radioactivity of DNP-glycine		Ratio	Radioactivity of fibroin (*c.p.m. per mg.*)
	N-terminal (*c.p.m. per* μM)	Non-N-terminal (*c.p.m. per* μM)		
20	95.4	59.8	1.55	148
60	61.3	103.8	0.45	199

1) Each silkworm was injected with 10^5 c.p.m. of DL-alanine-1-C^{14}

TABLE III

Comparison of Radioactiviy between N-terminal and Non-N-terminal Glycine of Fibroin Prepared by the TCA Method

Time after injection[1] (*hours*)	Radioactivity of DNP-glycine		Ratio	Radioactivity of fibroin (*c.p.m. per mg.*)
	N-terminal (*c.p.m. per* μM)	Non-N-terminal (*c.p.m. per* μM)		
0.5	44.9	9.4	4.80	10
1.5	86.4	67.9	1.27	121
4.0	55.2	217	0.25	453
18.0	0	130	0	315

1) Each silkworm was injected with 6×10^4 c.p.m. of glycine-1-C^{14}

precursor protein in the process of fibroin synthesis, because of that there was a lag phase during the time course of incorporation of radioactive amino acids into fibroin prepared by the TCA method, and, furthermore, the tissue protein had a radioactivity higher than that of fibroin at the early stage of the amino

TABLE IV

Specific Radioactivity of the Proteins[2] *Prepared from Silk-gland Tissue*

Time after injection[1] (*hours*)	Radioactivity of protein (*c.p.m. per mg.*)					
	Original tissue protein	Fraction I	Fraction II	Fraction III	Fraction IV	Fibroin
0.5	63	15	0	37	1495	10
1.0	122	58	3.5	156	2400	20
1.5	100	24	0	64	573	121
4.0	40	14	0	50	298	453
18.0	20	14	0	16	11	315

1) Each silkworm was injected with 6×10^4 c.p.m. of glycine-1-C^{14}.
2) See Scheme 1.

acid incorporation. It was therefore attempted to fractionate the tissue protein by the cupric ethylenediamine method as shown in Scheme 1. The radioactivity of each fraction is given in Tables IV and V. It was most remarkable that Fraction IV had a very high activity at 30 and 50 minutes after the injection. Silk fibroin was treated in the same manner, and confirmed to come into Fraction IV. It is thus likely that the protein of Fraction IV has a similar properties to fibroin. This fraction amounted to more than ten per cent of the starting tissuse protein.

The protein of Fraction IV was analyzed for the mode of labeling of glycine-C^{14} residue. The result is given in Table VI. The N-terminal glycine was found to be more than seven times as radioactive as non-N-terminal glycine.

TABLE V

Distribution of Radioactivity in the Tissue Protein

Protein	Total weight (*mg.*)	Radioactivity (*c.p.m. per mg.*)	Total radioactivity (*c.p.m.*)
Original tissue protein	200	122	24400
Fraction I	101	58	5858
,, II	54	3.5	189
,, III	21	156	3276
,, IV	23	2400	55200

The tissue protein of one hour experiment in Table IV was used.

TABLE VI

Specific Radioactivity of N-terminal and Non-N-terminal Glycine of Fraction IV[1)]

DNP-glycine	Radioactivity (*c.p.m. per μM*)
N-terminal	7440
Non-N-terminal	984
Ratio	7.55

1) Prepared from the tissue protein of one hour experiment in Table IV.

DISCUSSION

From the data presented in Tables I, II, and III it can be seen that the ratios of radioactivity of N-terminal glycine to that of non-N-terminal glycine change considerably with time after the injection of labeled amino acids. The data, showing a higher activity of N-terminal residues shortly after the injection, might be interpreted as indicating the combination of radioactive amion acid with peptide intermediates. As the amount of radioactive amino acid in the blood of the silkworm decreases with time the activity of N-terminal amino acid falls and approaches to zero, while non-N-terminal glycine remained to show a considerable high activity during 18 hours. These results agree with the findings of Gehrmann *et al.* (*14*) who observed that the specific radioactivity of C-terminal glycine residue of rat collagen was much lower than that of non-terminal glycine residues (approximately 1:3.3). In their experiment the rats were sacrificed 56 hours after the last injection of glycine-2-C^{14}.

The data in Table III are more clear-cut than those in Tables I and II in respect of non-uniform labeling. This difference is probably due to the preparating method of fibroin. The fibroin prepared by Griffin's method might have been contaminated with the precursor protein in the tissue. The TCA method is, therefore, advantageous over Griffin's method in obtaining pure fibroin from the posterior silkgland.

As mentioned above, the protein contained in Fraction IV has been assumed to be a precursor protein. However, it is not yet clear whether the protein is a true precursor protein or newly synthesized fibroin, which is just before secretion. The characteristic of the protein is under investigation. It might be noted here, however, that there is more than a seven-fold difference of radioactivity between N-terminal and non-N-terminal residues. As shown in Table V, more than 80 per cent of the total activity of the tissue protein was found in Fraction IV. This indicates that the Fraction IV protein is a main intermediate or product in the pathway of fibroin synthesis.

All the findings presented here seem to support the inference that silk fibroin is synthesized by a stepwise mechanism.

SUMMARY

1. An attempt was made to elucidate the mode of labeling of glycine and alanine residues in silk fibroin.

2. Silkworms were injected with glycine-1-C^{14} or alanine-1-C^{14} and radioactive fibroin was prepared from the posterior silkgland.

3. It was observed that, shortly after the injection, the specific activity of the N-terminal amino acid residues of the fibroin was two to five times as strong as the specific activity of the non-N-terminal amino acid residues. However, the former decreased more rapidly with time than the latter.

4. A protein having a considerably high radioactivity was separated from the tissue of posterior silkgland. The N-terminal glycine of this protein was also about seven times as radioactive as non-N-terminal glycine.

5. These results support the inference that the synthesis of fibroin occurs by a stepwise mechanism.

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STUDIES ON AMYLASE FORMATION BY BACILLUS SUBTILIS

VII. EFFECT OF PURINE, PYRIMIDINE AND THEIR ANALOGUES ON EXOENZYME FORMATION BY URACIL- AND ADENINE-REQUIRING MUTANTS

By JUNKO HOSODA, MASAMICHI KOHIYAMA AND MASAYASU NOMURA

(*From the Division of Enzymology, Institute of Applied Microbiology, University of Tokyo, Tokyo*)

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It was demonstrated in a previous paper (*1*) that amylase formation by *Bacillus subtilis* was inhibited by 8-azaguanine, and its inhibition was prevented by simultaneous addition of guanine or 2,4-dinitrophenol. The present investigation deals with the effects of nucleic acid-components and their analogues on the exoenzyme formation by purine- and pyrimidine-requiring mutants of *Bacillus subtilis.* The results extended and comfirmed the earlier conclusions gained from the experiments with the wild type strain of *B. subtilis.*

MATERIALS AND METHODS

Mutant strains of *Bacillus subtilis*, K12-2 (uracil-dependent), K10 (adenine-dependent) and K6011 (tryptophan-dependent and adenine-or hypoxanthine-dependent) were isolated by one of the authors* from an amylase producing strain, *B. subtilis* K. Unless otherwise stated experiments with stationary phase cells were carried out as follows: bacteria were grown aerobically in a broth for one day at 30° which were used as inocula. The inoculum was diluted into a hundred volumes of SA medium supplemented with 100 to 300 μg. of the required bases, grown aerobically for about 20 hours at 30°. Cells were harvested, washed once or twice with buffer and resuspended in fresh S medium (see below) in Monod's shaken tubes or test tubes containing testing materials. The reaction mixtures were incubated with shaking at 30°, aliquots were withdrawn at different time intervals and enzyme activity and optical density were measured.

The compositions of media were as follows: S-medium; soluble starch 8 per cent, Na-citrate 0.04 *M*, $(NH_4)_2HPO_4$ 0.15 *M*, KCl 0.02 *M*, $MgSO_4 \cdot 7H_2O$ 0.002 *M*, $CaCl_2$ 0.001 *M* and ethyl alcohol 1 per cent (*v/v*): SA-medium; S-medium plus 0.1 per cent casein acid-hydrolyzate: S_{-S} medium; S medium minus starch and ethanol. All of the media were adjusted to pH 7.2 with HCl or NaOH. In the case where either S or SA medium was used for resuspension, starch was hydrolized in advance by amylase, and then the amylase was inactivated by boiling. Amylase was assayed by the method of Hagihara (*2*) and protease was assayed by the method of Chaney and Tomarelli (*3*). Turbidity of cultures was determined nepherometrically or by measuring optical density at 550 mμ. 8-Azaguanine and 2-thiouracil are the products of Tanabe Pharmaceutical Company and Bayer Co. respectively.

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* M. Kohiyama.

RESULTS

It was previously reported that amylase formation by *Bacillus subtilis* initiated just after the end of logarithmic growth and amylase was produced linearly during the stationary phase. A considerable amount of protease(s) and ribonuclease were also produced in this phase. The purine- and pyrimidine-dependent mutants studied here were shown to grow and produce amylase in the stationary phase in the same amount as the wild type strain if supplied with sufficient amounts of required bases (Table I).

Effect of Purine or Pyrimidine on the Formation of Exoenzymes by the Stationary Phase Cells—Numerous reports have appeared in the literature which indicated that the biological synthesis of protein is dependent upon the synthesis (or metabolism) of nucleic acid (*4*, *5*). If continuous synthesis of nucleic acid is essential for amylase formation, the removal of the nucleic acid components, purine or pyrimidine, from the media should stop the amylase formation by auxotrophs which require these compounds for growth. This hypothesis was testified by the use of stationary phase cells of strains K12-2, K10 and K6011. The cells harvested from a stationary phase culture, washed and resuspended in fresh medium, produced considerable amount of amylase as well as protease lineary even in the absence of requiring bases for several hours. Meanwhile, in most experiments, addition of requiring bases had no effect on enzyme formation for one to two hours. After three hours, when the rate of enzyme production without required bases began to decrease, added

TABLE I

Growth and Amylase Formation of Bacillus subtilis K, KIO (adenineless) and KI2-2 (uracilless)

Strain	K		K 10		K 12–2	
Additions	—		Adenine sulfate 100 μg./ml.		Uracil 100 μg./ml.	
Incubation time (*hrs.*)	OD^{600}	Amylase	OD^{600}	Amylase	OD^{600}	Amylase
22	3.8	700	3.3	490	1.7	280
29	3.8	880	3.0	620	2.4	500
54	3.8	1650	3.0	1100	3.0	1250

Bacteria were grown in a broth for 24 hours with aeration. Inocula were added to 100 volumes of fresh SA media fortified with required bases as indicated in the table. The cells were grown further aerobically with shaking at 30°. Aliquots were withdrawn at intervals and amylase activity and optical density were measured.

bases stimulated enzyme formation as shown in Fig. 1. The extent of stimulation of required bases varied from experiment to experiment, and in several cases no stimulation resulted during five hours' incubation. In general, the stimulation upon protease formation was not so significant as that on amylase formation.

Effect of Uracil, 8-Azaguanine and 2,4-Dinitrophenol on Amylase and Protease Formation by the Uracil-dependent Strain—The data presented in the previous section provoke a question that by what mechanism the enzyme are formed in the absence of uracil or adenine. Is it on account of a) the presence of prototroph derived by reversion b) the presence of preformed nucleic acid or c) new synthesis of nucleic acid from purine- and pyrimidine-pool within the cells? The first possibility was excluded by the fact that the revertant frequency was found to be less than 10^{-8} in the experimental condition for all three mutants. In order to examine other two possibilities the effect of 2,4-dinitrophenol and 8-azaguanine on the enzyme formation by the uracil-dependent mutant was studied. Typical results are shown in Fig. 1 and Table III. Amylase and protease were produced almost linearly for 3 hours without uracil. Then the rate of enzyme synthesis decreased owing probably to partial cell lysis which was indicated by the marked decrease in optical dencity. The addition of uracil stimulated the enzyme formation after 3 hours, maintaining the initial level of turbidity of the culture. In the absence of uracil, 2,4-dinitrophenol presented no inhibitory effect on the enzyme formation as in the case of the enzyme formation by wild type strain as reported previously (*1*), while the stimulatory effect of added uracil upon the enzyme formation was suppressed. Azaguanine, when added alone, had no effect but if it was added in the presence of uracil, inhibited the enzyme formation almost instantly and completely. The more uracil was added the more the inhibition became serious. These results suggest that azaguanine disturbs the enzyme formation by taking part in the synthesis of unphysiological nucleic acid when other components of nucleic acid are available. Furthermore, it seems probable that the enzyme forming activity in the absence of uracil depends rather on the nucleic acid present at the time than on the synthesis of new nucleic acid from uracil pool.

Effect of Azaguanine on Adenine-dependent Mutant and Effect of Thiouracil on Uracildependent Strain—Azaguanine inhibited amylase formation by adenine-dependent mutants as shown in Table II. The inhibition seemed to be competitive with adenine. A fairly large amount of adenine was needed to overcome it. It was proved in another experiment that the inhibition by azaguanine on adenine-dependent mutant could be restored with adenine but not with guanine.

Thiouracil inhibited amylase formation by the uracil-dependent mutant as well as that by the adenine-dependent mutant. Thiouracil and uracil seem to be competitive in the case of uracil-dependent strain as in Table III. On the other hand, this inhibitor was found to be more effective on the enzyme formation by adenine-dependent mutant when it was added together with adenine. Thus the mechanism of inhibition by thiouracil seems to be similar to that of

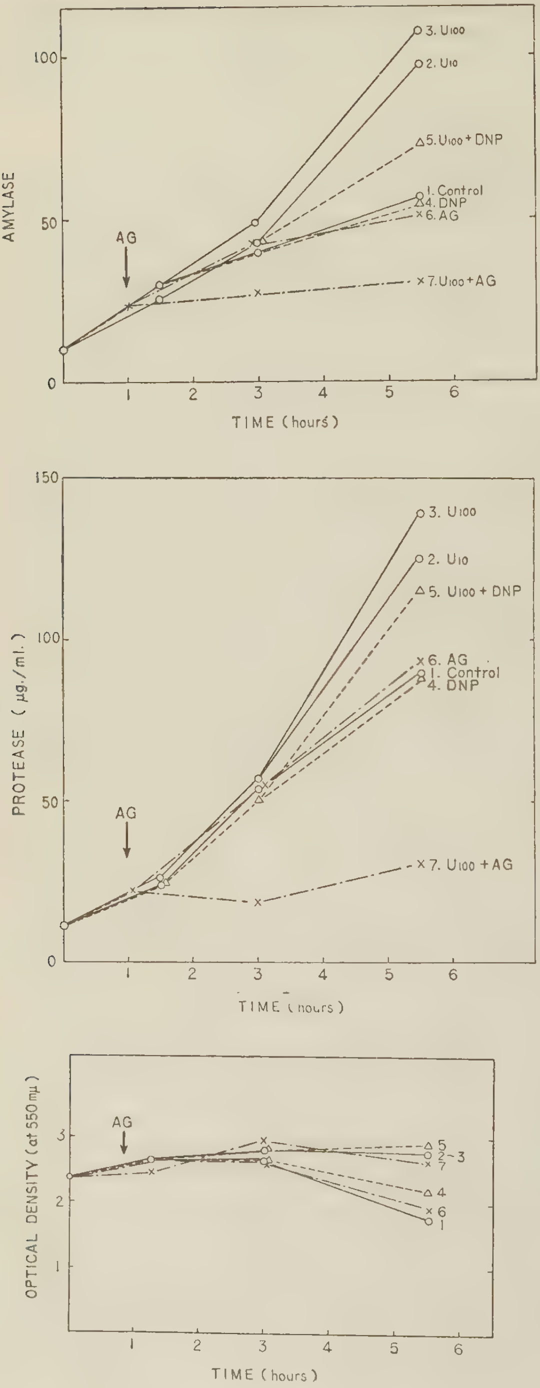

FIG. 1. The effect of uracil, DNP and azaguanine on amylase (a) and protease (b) formation and cell growth (c) of uracilless mutant of *B. subtillis* K12–2.

Stationary phase cells of K12–2 were resuspended in fresh S medium placed in Monod's shaken tube with further addition described as follows:

Curve 1, without addition; 2, 10 μg./ml. uracil; 3, 100 μg./ml. uracil; 4, 1×10^{-4} *M* DNP; 5, 10^{-4} *M* DNP plus 100 μg./ml. uracil; 6, 5×10^{-3} *M* azaguanine; 7, 5×10^{-3} *M* azaguanine plus 100 μg./ml. uracil. (Azaguanine was added after 1 hours' incubation).

The mixtures were incubated with shaking at 30° and aliquots were withdrawn for assay of enzyme activity and optical density.

azaguanine, although the degree of inhibition by thiouracil is always slighter than that by azaguanine in so far as the strains and the experimental conditions are concerned.

TABLE II

The Effect of 8-Azaguanine on Amylase Formation by K60II and Its Reversal by Adenine

Additions		Amylase (D 30′/60°)			Optical density (550 mμ)		
Adenine sulfate (*μg./ml.*)	Azaguanine ($\times 10^{-4}$ *M*)	After incubation (hrs.) 0	2	6	After incubation (hrs.) 0	2	6
0	—	6	23	78	2.6	3.1	3.1
5	—	6	23	82	2.6	3.3	3.0
100	—	6	23	118	2.6	3.0	3.3
0	4.5	6	13	14	2.6	—	3.0
5	4.5	6	14	15	2.6	—	2.9
100	4.5	6	21	60	2.6	3.3	4.3

The stationary phase cells of K6011 resuspended in SA medium supplemented with 100 μg./ml. of DL-tryptophan.

TABLE III

The Effect of Thiouracil and Azaguanine on Amylase Formation by KI2-2 (uracilless)

Addition			Amylase after incubation (hrs.) (D 30′/60°/*ml.*)			
Uracil (*μg./ml.*)	Thiouracil (*μg./ml.* (10^{-4} *M*))	Azaguanine (*μg./ml.* (10^{-4} *M*))	0	1.5	3	6
0	0	0	5.7	15	21	36
0	340 (25)	0	5.7	16	19	25
0	0	74 (5)	5.7	12	16	28
5	0	0	5.7	15	29	45
5	340	0	5.7	15	26	31
5	0	74	5.7	15	10.2	16.9
50	0	0	5.7	17	43	108
50	340	0	5.7	17	29	67
50	0	74	5.7	—	9.0	10

Conditions of culture and incubation were similar to those in Fig. 1 unless inhibitors were added at the start of incubation.

Effect of Carbon Sources—In the experiments described above high concentration of starch hydrolysate as well as citrate were added to the media for resuspension as carbon sources. It was because they were known to be stimulating upon amylase formation if contained in growth media. It was found, in the course of experiments, that if starch hydrolysate was omitted from the resuspension media, the required bases and the analogues did not affect enzyme formation at all. Therefore, the effect of carbon sources was examined (Table IV). Among the substances tested effect of starch hydrolysate and glucose seemed to be different from that of others and the effects of uracil and azaguanine appeared remarkably in the presence of these sugars. The formation of exoenzymes in the presence of organic acids was not affected by these bases and the amount of enzyme formed was always higher than that in the presence of carbohydrates.

Table IV

Effect of Carbon Sources on Activation and Inhibibion of Amylase Formation by K 12-2 (uracilless)

Exptl. No.	Carbon sources	Amylase (D30′/40°/*ml.**)					Protease (*µg./ml.*)				
		Before incubation	After 5 hours' incubation with				Before incubation	After 5 hours' incubation			
			No	AG	U	AG +U		No	AG	U	AG +U
1	Citrate	2.4	62	64	64	67	—	29	39	35	36
	Citrate plus glucose	2.4	36	36	67	8.3	—	37	35	45	7.5
	Citrate plus starch hydrolizate	2.4	39	29	44	10	—	49	42	120	14
2	Without carbon source	2.5	12		13						
	Citrate	2.5	28	26	28	26					
	Succinate	2.5	40	40	40	40					
	Acetate	2.5			45	45					
	Glucose	2.5	20	19	21	2.9					

The composition of resuspension medium was as follows: $(NH_4)_2HPO_4$ 0.1 *M*, $MgSO_4$ 0.002 *M*, KCl 0.01 *M*, $CaCl_2$ 0.001 *M* with 1 per cent starch hydrolyzate or 2 per cent glucose in Expt. 1, and 1 per cent carbon sources in Expt. 2 pH=7.3.

* In Expt. 2, incubation time was 4 hours.

DISCUSSION

A number of works have been taken to study the role of nucleic acid on protein synthesis using biochemical mutants of microorganisms which require nucleic acid components (purines and pyrimidines) for their growth. In the case of inducible enzyme the enzyme synthesis stopped when these growth factors were omitted from the incubation media *(4 5)*, and from the results it was concluded that the continuous synthesis of RNA is essential for the inducible enzyme synthesis. Similar conclusion has been drawn by using purine and pyrimidine analogues as inhibitor of nucleic acid synthesis (*5*, *6*). On the other hand the mechanism of amylase formation by *B. subtilis* has been shown to be different from that of inducible enzyme synthesis in several points (*7–9*). Present data with uracil-dependent mutant seem to confirm the previous conclusion of the role of RNA in amylase formation by a wild type strain of *B. subtilis* H (*1*).

The fact that the inhibition of azaguanine upon the formation of amylase and protease appears only the condition for the synthesis of RNA is more favorable indicates that this inhibition is rather due to the synthesis of unphysiological nucleic acid containing azaguanine than to inhibition of RNA synthesis. Enzyme forming activity without required bases was observed and this activity was not inhibited by 2,4-dinitrophenol at the concentration of 10^{-4} M which was known to inhibit the incorporation of P^{32}-phosphate and C^{14}-adenylic acid into the nucleic acid fraction of *B. subtilis* H (*1*). The promoting effect of uracil upon the formation of amylase and protease by the uracil-dependent mutant was suppressed by DNP. From these observations it may be concluded that the continuous synthesis of RNA from purine and pyrimidine level is not necessary for the exoenzyme formation but the amount of the RNA which participates the enzyme formation is important, and addition of purine or pyrimidine stimulates the enzyme forming activity when the amount of the RNA is not sufficient.

The inhibition of azaguanine on amylase formation by adenine- or hypoxanthine-dependent mutant was reversed by the addition of adenine into the medium. The possible explanations of this phenomenon are following: azaguanine may be incorporated into nucleic acid instead of adenine or guanine in the medium can hardly be assimilated by this auxotrophic mutant. But the conclusion must await further studies on the mechanism of RNA synthesis by this bacterium.

The effect of carbon sources upon the inhibition and stimulation by the bases was complicated. Synthesis of RNA from purine and pyrimidine requires ribose residue (PRPP) (*10*) but the organic acids are not so readily convertible to it as glucose, and the nucleic acid synthesis may not be taken place in the absence of sugars even if the bases are present. Creaser's observation (*6*) that glucose increased the stimulation of β-galactosidase formation by purines and pyrimidines and azaguanine produced a much larger inhibition in the presence of glucose than when glucose was omitted from the system and the amount of azaguanine incorporated into RNA was 60 per cent less when glucose was absent supports this view. Table IV shows that the sugars suppressed the promoting effect of organic acids on enzyme formation. In the course of this experiment it was observed that the turbidity of cell suspension decreased during the incubation in the presence of organic acids even if uracil was supplied, but the initial turbidity was maintained when sugars and uracil were present. It has been observed (*9*) that the growth of *B. subtilis* caused by addition of glucose to the resting cells by dilution of the culture diminished the amylase forming activity per unit cell of the bacteria. These facts suggest that the process of cell growth or maintenance of cellular mass competes with that of amylase and protease formation and the effects of carbon sources concern with the regulation of these mechanisms.

SUMMARY

The relation between enzyme (amylase and protease) formation and synthesis of RNA was studied using purine- and pyrimidine-requiring mutants of *Bacillus subtilis*.

1. Uracil-dependent and adenine-dependent mutants produced a considerable amounts of amylase and protease in the absence of the growth factors, though the factors stimulated more or less the enzyme formation.

2. 2,4-Dinitrophenol did not inhibit the enzyme formation in the absence of required components, while inhibited the promoting effect of these compounds.

3. Azaguanine did not inhibit the enzyme formation by uracil-dependent mutant in the absence of uracil in the medium, while its inhibition was almost instant and complete when uracil was present in the medium and the degree of inhibition depended upon the amount of uracil present.

4. Azaguanine inhibited the enzyme formation by adenine- or hypoxanthine-requiring mutant and this inhibition was reversed by addition of adenine.

5. Thiouracil inhibited the amylase formation by uracil-dependent mutant and the inhibition was diminished when uracil was present.

6. The effects of carbon sources on the enzyme formation by the mutant bacteria were studied and discussed.

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HEMOPROTEINS OF WHEAT GERM*

I. CRYSTALLIZATION AND PROPERTIES OF PEROXIDASE FROM WHEAT GERM

By KUNIO TAGAWA AND MASATERU SHIN

(*From the Department of Biology, Faculty of Science, University of Osaka, Osaka*)

(Received for publication, November 13, 1958)

It was previously reported that cytochrome c and two hemoproteins with reduced α-bands at 556 mμ and 566 mμ respectively were obtained in crystalline form from wheat germ (*1–3*). These and especially hemoprotein 556, may be related to peroxidase because of the strong peroxidatic activity and of the absorption specta obtained on addition of various reagents.

In the investigations described in this paper, these two pigments were found to complex with hydrogen peroxide, and not to be reduced by physiological electron donors in the presence of an active homogenate or particles from wheat germ. It is, therefore, concluded that these pigments are preoxidases. In the present and succeeding papers, therefore, the names peroxidase 556 and 566 will be used for these hemoproteins.

The hemoprotein samples prepared according to the method described previously (*3*), in which the ethylacetate and acid treatments were employed, contained mostly preoxidase 556 and very little peroxidase 566. By the present method, avoiding such drastic treatments, however, much more peroxidase 566, which seems to be the native peroxidase, and less peroxidase 556 was obtained

This paper describes the new method for the preparation and some properties of peroxidase from wheat germ.

MATERIALS AND METHODS

Wheat Germ—Canadian wheat (Manitoba-3) was used. This pure wheat germ contained about 10 per cent moisture, and was kindly supplied by Nisshin Flour Milling Co. Ltd.

Measurement of Absorption Spectra—Quantitative determinations were made in a Shimazu spectrophotometer, type QB-50, and a Beckman-DK model. The absorption spectra of the peroxidase-hydrogen peroxide complexes were also studied using a rapid scanning spectrophotometer.

Measurement of Peroxidatic Activities—The peroxidatic activities were measured spectrophotometrically by following the oxidation of hydrogen donors such as pyrogallol and guaiacol in the presence of hydrogen peroxide and the enzyme. The measurement

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* This investigation was supprted in part by a research grant (No. RG-5871) from the National Institutes of Health, United States Public Health Service.

was also made by the benzidine-ascorbic acid-test (*4*) in which a definite amount of ascorbic acid was oxidized in the presence of hydrogen peroxide, benzidine and the enzyme.

Purification and Crystallization of Peroxidase 566

Step 1. Extraction and Ammonium Sulfate Precipitation—Wheat germ was extracted with 5 volumes of 0.1 *M* phosphate buffer of pH 6.5 for 1 hour at room temperature. To the slightly turbid yellow extract solid ammonium sulfate was added to 72 per cent saturation. The resulting precipitate was collected on a Buchner funnel and then pressed in a cloth to drain out the ammonium sulfate solution.

Step 2. Fractionation with Ammonium Sulfate—The precipitate was suspended in 4 volumes of cold water. After centrifugation of the suspension, solid ammonium sulfate was added to the turbid supernatant to 60 per cent saturation (specific gravity, 1.162). The precipitate formed was collected on a Buchner funnel and drained. The filtrate contained no peroxidase but most of the cytochrome c and was used for the preparation of the latter (*1. 2*). The precipitate containing the bulk of the peroxidase was resuspended in cold water and dialysed against ice cold tap water over night. The large amount of brown precipitate formed was filtered off, and the filtrate was then redialysed.

Step 3. Adsorption on Resin—The dialysed peroxidase solution was passed through an Amberlite CG-50 resin column equilibrated with 0.02 *N* ammonium phosphate buffer, pH 6.5, and the column was washed with the same buffer. The portion of the resin which had adsorbed the peroxidase was suspended in the same buffer. After adjusting the pH to 7.0 with ammonia, and eluting the resin with 0.25 *N* buffer, pH 7.0, the resulting eluate was dialysed and readsorbed on XE-64 resin. The peroxidase adsorbed on the resin was first eluted with 0.15 *N* buffer and then with 0.25 *N* buffer of pH 7.5.

Step 4. Chromatography—Both eluates obtained in Step 3 were chromatographed separately on XE-64 resin columns using 0.075 *N* and 0.10 *N* ammonium phosphate buffer of pH 7.0, respectively.

Step 5. Crystallization—From the main effluent of these two chromatographic treatments, crystalline peroxidase was obtained as follows: The fraction precipitated by 45 to 60 per cent saturation of ammonium sulfate was dissolved in a minimum of water, and then saturated ammonium sulfate was added until the solution became slightly turbid. Crystals of peroxidase appeared after a few days.

RESULTS AND DISCUSSION

Preparation Procedure

Extraction and Relation Between Peroxidase 556 and 566—Organic solvents have been employed for pretreatment prior to the extraction of cytochrome c from wheat germ (*1*, *2*, *5*). Ethylacetate was previously employed prior to the extraction of peroxidase (*3*). Both peroxidase 556 and 566 were present in the extract but the latter in very small amount. By the present method, however, it was found that most of the peroxidase could be extracted with 0.1 *M* phosphate buffer, pH 6.5, even without pretreatment by organic solvents, and that peroxidase in the extract was largely 566, little peroxidase 556 being obtained. To examine the effect of pretreatment and extraction on the peroxidase the following experiments were carried out: 10 g. batches of wheat germ were washed for 30 minutes in a column with various organic solvents and then dried in air. After extracting with 40 ml. of each agent, the extracts were centrifuged for 20 minutes at 8,000×*g*. The amount of peroxidase in each extract was compared in a spectroscope after reduction with sodium dithionite. The results are summarized in Table I.

TABLE I

Effect of Reagents on Extraction of Peroxidase from Wheat Germ

Pretreatment	Extraction	Intensity of absorption maxima of reduced form		
		550 mμ	556 mμ	566 mμ
None	0.1 *M* phosphate, pH 6.5	++	−	++
,,	30% sat. Am_2SO_4*, pH 6.5	+++	−	++
Acetone	0.1 *M* phosphate, pH 6.5	++++	+	+++++
,,	30% sat. Am_2SO_4*, pH 6.5	++++	+	+++++
Alcohol	0.1 *M* phosphate, pH 6.5	+	+	+
,,	30% sat. Am_2SO_4*, pH 6.5	++	+	++
Ethylacetate	0.1 *M* phosphate, pH 6.5	+++++	++	++++
,,	30% sat. Am_2SO_4*, pH 6.5	+++++	++	++++

* Am_2SO_4 represents ammonium sulfate.

The amount of both peroxidases extracted without pretreatment was less than that after pretreatment with acetone or ethylacetate. It was noticed that the amount of peroxidase 556 in the extracts which had been pretreated with organic solvents increased gradually on standing in the cold room. The rate of increase was much greater in acidic medium. Spectroscopic examination of wheat germ paste which had been reduced with sodium dithionite, however, showed no strong band around 556 mμ in contrast to a strong broad band at 560–570 mμ (Fig. 1). It is, therefore, supposed that peroxidase 556 is absent in the native tissue and is produced by some sort of structural modifications during treatment with organic solvents. Therefore in the present method, the ethylacetate and acid treatments used in the previous method (*3*) were omitted, although this reduced the yield of peroxidase.

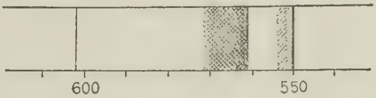

FIG. 1. Absorption band of reduced wheat germ. Observation was made on wheat germ paste after reduction with dithionite.

Horseradish peroxidase forms complexes with acids and alkali, which can be distinguished spectrophotometrically (*6–8*). Peroxidase 556 behaves in the same way. However, peroxidase 566 forms no complex in the ferric form with any agents within pH range for stability (*3*), but in acidic medium spectrophotometrical changes are observed. At acidic pH the enzyme solution became turbid owing to denaturation of the enzyme protein.

Theorell (*9*) had obtained two preparations of peroxidase from horseradish, one of which (peroxidase I) showed the hemochromogen bands at 565 mμ and 535 mμ, and other, peroxidase II, was like methemoglobin. Keilin and Hartree (*10*) reported that peroxidase I was formed from peroxidase II during preservation in the cold room, and that some of its complexes with various reagents showed the same spectra as those of peroxidase II. It is thought from the results described above that peroxidase I and II correspond to our peroxidase 566 and 556, respectively, but contradictorily to the report of British researchers the latter is formed from the former.

Fractionation with Ammonium Sulfate—In the previous method, the first extract of wheat germ was precipitated with 95 per cent ammonium sulfate, treated with acetic acid, and then fractionated with ammonium sulfate into peroxidase and cytochrome c fractions precipitating with 40–75 and 75–95 per cent saturation, respectively.

In the present method, the first precipitation with ammonium sulfate was carried out at 72 per cent saturation and this precipitate was fractionated further by 30–60 and 60–95 per cent saturation, respectively. This difference in the concentration of ammonium sulfate required for salting out might be due to the presence of more impurities in the extract obtained by the new method, in which ethylacetate and acid treatments were omitted. This is likely because the purified peroxidase 566 obtained by either method was completely precipitated by 65 per cent ammonium sulfate.

Adsorption and Chromatography on Resin—In the first eluate at Step 3 only peroxidase 566 was found, whereas, in the passed fraction obtained by the first adsorption procedure (Step 3), little peroxidase 556 was present (as judged by the strength of the hemochromogen band at 556 mμ after addition of ammonia and dithionite). However, no peroxidase 556 was found in later fractions using the present method.

In the previous method more than 10 peroxidase bands appeared on column chromatography. These may have been formed by various modifications of the peroxidase caused during the treatment with ethylacetate and acid. In the present method, since only three or four bands of peroxidase appeared during the second adsorption and washing procedure at Step 3, and since these were completely eluted with 0.25 N phosphate buffer, pH 7.0, following chromatographic steps were made on two resin columns buffered with 0.075 N and 0.1 N buffer, pH 7.0, respectively. In the previous method, gradient development was made from 0.05 N to 0.2 N buffer (*3*).

Fig. 2 shows typical chromatograms obtained by the present method in which each peroxidase sample is divided into two fractions. From both Fraction II and III in Fig. 2 peroxidase 566 could be crystallized (Fig. 3).

In the preparation of cytochrome c, chromatogrophy on a resin column

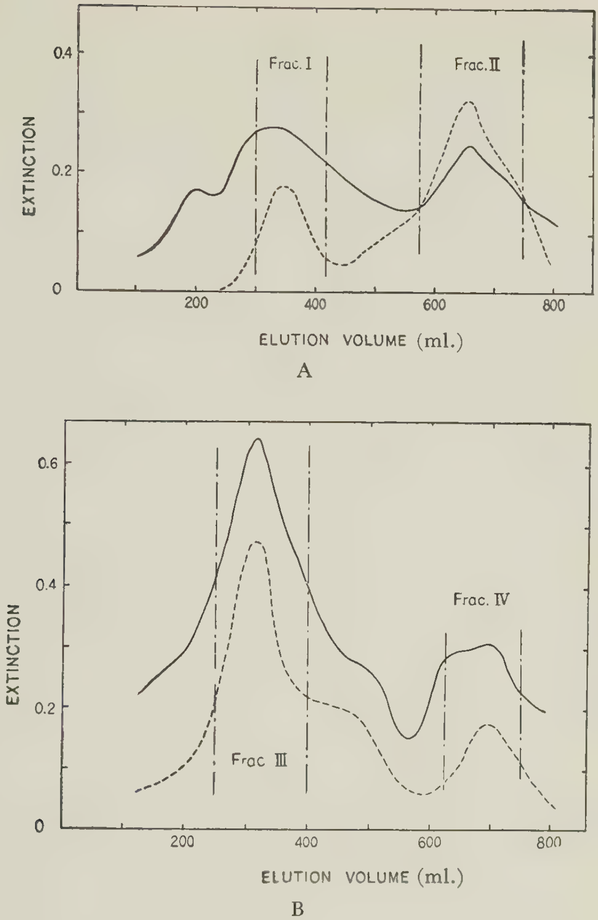

FIG. 2. Chromatograms of peroxidase 566 of wheat germ.

A. Chromatogram of eluate with $0.15\,N$ ammonium phosphate buffer by the second elution procedure (Step 3). Chromatography was carried out on an Amberlite EX-64 resin column (3×30 cm.) using $0.075\,N$ ammonium phosphate buffer, pH 7.0. Solid line; extinction at 278 mμ, dotted line; extinction at 417 mμ.

B. Chromatogram of eluate with $0.25\,N$ ammonium phosphate buffer by second elution procedure (Step 3). Chromatography was done in the same way as A except that $0.10\,N$ ammonium phosphate buffer was used.

has revealed only one band.

However, in the case of peroxidase 566, three or four bands appeared on the resin column. It is, therefore, unknown which of these fractions corresponds to the native form, as discussed in our preceding papers dealt with the preparation of cytochrome c (*11–13*).

Yield and Purity—Both crystalline peroxide 556 and 566 prepared according

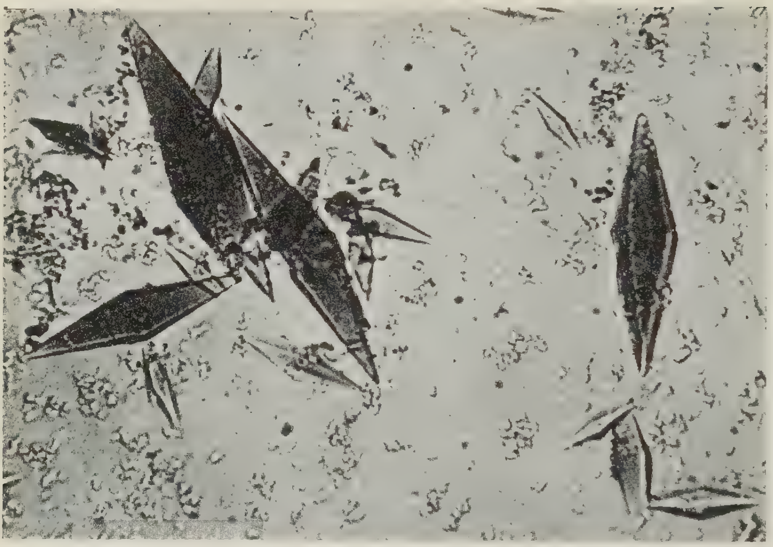

FIG. 3. Crystalline peroxidase 566 of wheat germ (Octahedron, ×250).

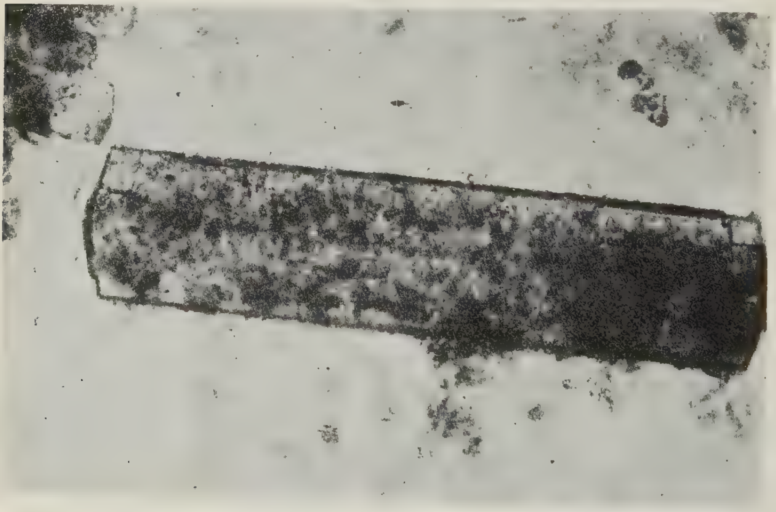

FIG. 4. Crystalline peroxidase 556 of wheat germ (plate form, ×150).

to the previous method showed extinction ratios, R^{411}_{278}, of 4.5 and R^{417}_{278} of 1.2. The crystals from Franction II and III in the present method have R^{417}_{278} ratios of 1.3 and 0.8*, respectively. Both compounds have the same prosthetic group, *i.e.*, protohematin (identified by the absorption spectra of the pyridin-hemochromogen). Therefore the remarkable difference in extinction ratios of the peroxidases may indicate that the protein moiety of peroxidase 566 is considerably different from that of peroxidase 556.

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* The lower value of R^{417}_{278} of Fraction III may be due to protein contamination.

The yield of crystalline peroxidase 566 from 2 kg. of wheat germ was about 40 mg. and less than 10 mg. in the present and previous methods, respectively.

Properties—Absorption Spectra of Peroxidases and Their Derivertives: Absorption spectra of both peroxidases and their complexes with cyanide, fluoride, azide, alkali and carbon monoxide have already been described (*3*). Both peroxidases also formed complexes with hydrogen peroxide. Their absorption spectra in the visible region were recorded using a rapid scanning spectrophotometer (Fig. 5, 6). As illustrated in Fig. 5, the peroxidase 556-hydrogen peroxide complex has two shoulders at 520–530 $m\mu$ and 550–560 $m\mu$, which are very labile and had almost disappeared after 5 minutes. It appears to us that this compound corresponds to Complex II (*10*, *14*).

Absorption curves of the peroxidase 566-hydrogen peroxide complex are shown in Fig. 6. In this case, very distinct peaks appeared at around 540 $m\mu$ and 570 $m\mu$ 1 minute after the addition of hydrogen peroxide, and remained

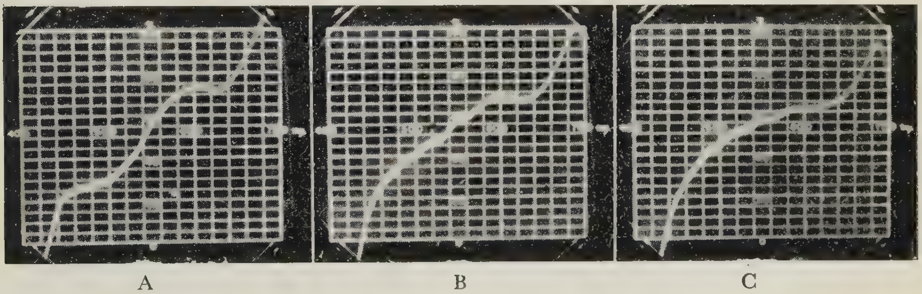

FIG. 5. Absorption spectra of peroxidase 556-hydrogen peroxide complex.

Reaction mixture contained 0.06 *M* phosphate buffer pH 6.0, 100 μM H_2O_2 and peroxidase 556.

A: Absorption spectrum of peroxidase 556.
B: Absorption spectrum of the complex after 1 minute.
C: Absorption spectrum of the complex after 5 minutes.

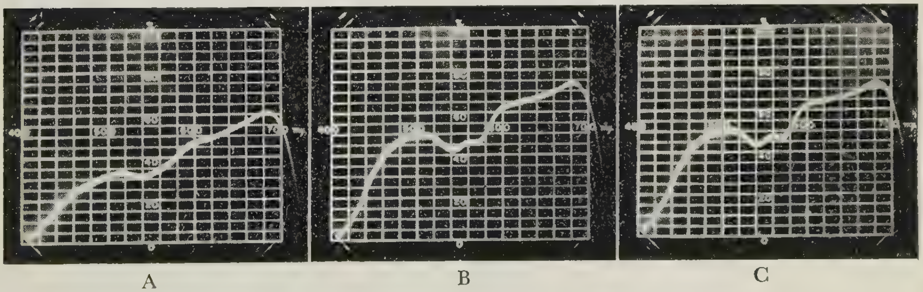

FIG. 6. Absorption spectra of peroxidase 566-hydrogen peroxide complex.

A: Reaction mixture contained 0.06 *M* phosphate buffer, pH 6.0, 0.005 *M* H_2O_2 and peroxidase 566.
B: Absorption spectrum of the complex after 1 minute.
C: Absorption spectrum of the complex after 30 minutes.

for more than 20 minutes. This compound is not identical to any of the known complexes (*10*), but available evidence favors the assumption that this is a true peroxidase-hydrogen peroxide complex.

Peroxidatic Activities: The peroxidase reaction was shown as follows;

$$\mathrm{E+S} \xrightarrow{k_1} \mathrm{ES}$$

$$\mathrm{ES+AH_2} \xrightarrow{k_4} \mathrm{E+P}$$

It had been shown by Chance (*15*) that the activity of the peroxidase reaction should be estimated from the k_4 value, and a hydrogen donor (AH_2) must be chosen to establish the relation, that $k_4 a \ll k_1 x$, where a and x represent the concentration of AH_2 and peroxide (S), respectively, to facilitate the kinetic study. For this purpose, pyrogallol, benzidine and guaiacol were studied as hydrogen donors. However, it was not possible in any test to obtain a zero order reaction in relation to the concentration of hydrogen peroxide. Therefore, it is not possible to estimate the activities of wheat peroxidases by kinetic studies at present. A suitable method for the determination of the activity will be devised later. However, it is roughly estimated that both peroxidases possess about half the activity of crystalline Japanese radish peroxidase, which has a P. N. of 1730 (*16*).

SUMMARY

A new method is described for purification and crystallization of peroxidase from wheat germ. In this method the peroxidase is directly extracted without treatment with organic solvents and acetic acid. It is adsorbed on Amberlite CG-50 resin equilibrated at pH 6.5 and then eluted with a buffer of pH 7.0.

The eluted peroxidase is again adsorbed on XE-64 resin and separates into two fractions during elution. These fractions are chromatographed on different resin columns using buffers of 0.075 *N* and 1.0 *N*, respectively. Crystalline peroxidase is obtained from both main fractions.

In this method peroxidase 566, the native peroxidase, is obtained in larger amount than in the previous method in which peroxidase 556, a modified form was the chief peroxidase.

Peroxidase 566 forms a very stable hydrogen peroxidase complex which is not identifiable with any of the established complexes.

The authors are greatly indebted to Prof. K. Okunuki and Dr. B. Hagihara for their kind advice during this work.

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HEMOPROTEINS OF WHEAT GERM*

II. PARTICULATE ELECTRON TRANSPORTING SYSTEM OF WHEAT GERM AND THE EFFECT OF PEROXIDASE ON IT

By KUNIO TAGAWA and MASATERU SHIN

(*From the Department of Biology,*
Faculty of Science, University of Osaka, Osaka)

(Received for publication, November 13, 1958)

Horse radish has been used as a good source of plant peroxidase (*1*, *2*), but wheat root (*3*) and germ (*4*, *5*) also contain considerable quantity of this enzyme. From wheat germ we have obtained two types of peroxidase in crystalline form (*4*, *5*). Although peroxidase is capable of catalyzing the oxidation of various hydrogen donors in the presence of peroxide, its role in cell metabolism is not known.

Recently, Lundegårdh (*3*) reported that " cytochrome dh " is a reduced peroxidase complex. It is of interest, therefore, to study its connection with the cytochrome system. We could demonstrate an effect of peroxidase on the cytochrome system, but failed to obtain a direct evidence that peroxidase is a component of the cytochrome system and capable of transporting electrons from a donor. The present paper reports on the effect of peroxidase upon the electron transport system in isolated particles from wheat germ.

MATERIALS AND METHODS

Preparation of Particles from Wheat Germ—Wheat germ (20 g.) was soaked in 40 ml. of water and incubated for 2 hours at 20°. To the soaked germ 20 ml. of 0.4 *M* sucrose and 0.2 *M* potassium phosphate buffer, pH 7.0, were added. After chilling to 0°, it was homogenized in a blendor for 30 seconds. The homogenate was filtered through gauze and then centrifuged at $3,000 \times g$ for 10 minutes. The turbid supernatant was centrifuged at $18,000 \times g$ for 20 minutes and the pellet was washed twice with 40 ml. of 0.2 *M* sucrose and 0.1 *M* phosphate buffer, pH 7.4. It was then suspended in 20 ml. of the same mixture. The resulting suspension contained about 0.3 to 0.5 mg. of nitrogen per ml.. It was used in the present work as the particle preparation.

Peroxidase—Both peroxidases 556 and 566 were prepared by the method described previously (*4*, *5*).

Antimycin A—The material was purchased from the Kyowa Hakko Co., Ltd.

Cytochrome c—The crystalline oxidized cytochrome c was prepared from cow heart muscle according to the method used in this laboratory (*6*, *7*).

DPN and DPNH—Highly purified DPN was prepared from baker's yeast (*8*). DPNH

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* This investigation was supported in part by a research grant (No. RG-5871) from the National Institutes of Health, United States Public Health Service.

was prepared from this DPN, by an enzymic method (*9*).

Assay of Enzyme Activities—Reduction of cytochrome c was determined spectrophotometrically at 550 $m\mu$ in the presence of $10^{-4}\,M$ cyanide or under anaerobic condition. Oxidation of DPNH was measured by following the decrease in extinction at 340 $m\mu$.

Observation of Absorption Bands—Absorption bands of wheat germ homogenates and particulate suspensions were studied with a microspectroscope equiped with a strong light source.

RESULTS

Absorption Bands—Fig. 1 shows that the homogenate of wheat germ has cytochrome a, b and c components and is capable of oxidizing DPNH and succinate *via* the cytochrome system. The strong broad band which appeared at 560 to 570 $m\mu$ after reduction with dithionite represents the peroxidase and cytochrome b components (Fig. 1, C), but the former was scarcely reduced by substrates under completely anaerobic conditions (Fig. 1, A, B). When either peroxidase 566 or 556 was added, they were not reduced by either the homogenate or particles from the germ (Fig. 2). Unlike the particles from another

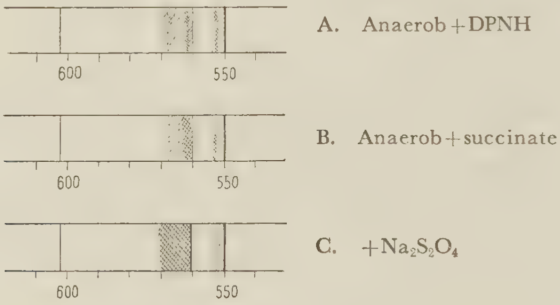


FIG. 1. Visible absorption bands of wheat germ. In A and B, absorption bands were observed anaerobically after incubation with DPNH and succinate, respectively. In C, they were observed after reduction with $Na_2S_2O_4$.

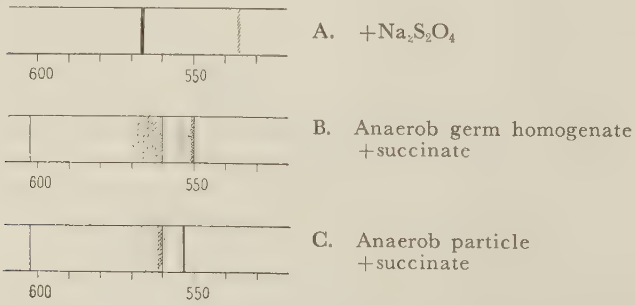

A. $+Na_2S_2O_4$
600
550
B. Anaerob germ homogenate +succinate
600
550
C. Anaerob particle +succinate
600
550

FIG. 2. Visible absorption bands of peroxidase of wheat germ. A shows absorption bands of reduced peroxidase 566. In B and C, peroxidase 556 or 566 was incubated anaerobically with succinate in a homogenate or particulate suspension, respectively.

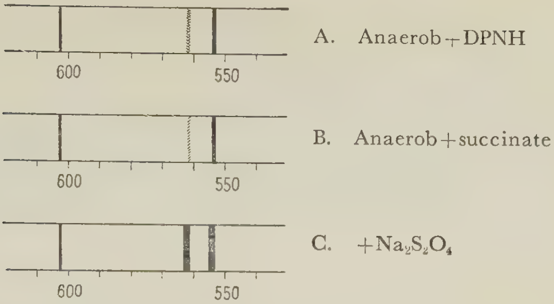


FIG. 3. Visible absorption bands of isolated particles from wheat germ. Observations were made in the same way as in Fig. 1.

sources, the particles from wheat germ showed distinct bands of cytochrome a, b and c_1 but not of cytochrome c after reduction with dithionite or in the presence of substrate under anaerobic conditions (Fig. 3).

Reduction of Cytochrome c—The particles from wheat germ catalyzed the reduction of added cytochrome c by DPNH or succinate in the presence of 10^{-4} M cyanide. The rate of the reaction with DPNH was about three times as fast as that with succinate. The latter reaction was almost completely

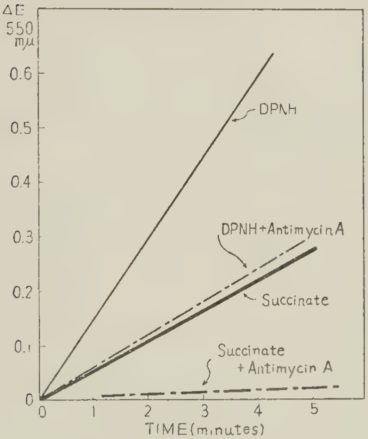


FIG. 4. Reduction of cytochrome c with DPNH and succinate in a particulate suspension from wheat germ.

Reaction mixture contained 0.1 M sucrose, 0.05 M phosphate buffer, pH 7.4, 1×10^{-4} M DPNH or 0.005 M succinate, 1×10^{-4} M KCN, 5×10^{-5} M cow heart cytochrome c and a particulate suspension containing 90 μg. nitrogen per ml. in total volume of 4 ml.. 30 μg. per ml. of antimycin A was present in some reaction mixtures as indicated in the figure.

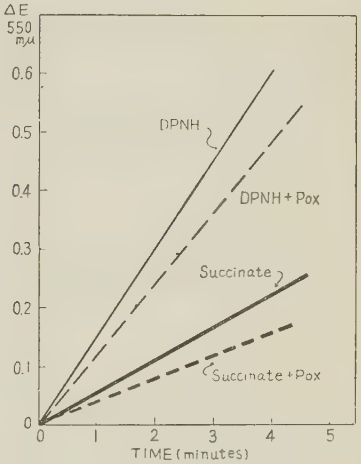


FIG. 5. Effect of peroxidase on reduction of cytochrome c with DPNH and succinate in a particulate suspension.

About 0.02 per cent peroxidase 556 or 566 was present in the reaction mixtures indicated in the figure. Other reagents were the same as in Fig. 4. Pox in the figure represents peroxidase.

inhibited by antimycin A, whereas the former reaction was inhibited only about 65 per cent by it (Fig. 4, Table I). This shows that the reduction of cytochrome c with succinate proceeds *via* cytochrome b.

Although all of the electrons do not seem to be transported *via* cytochrome b in the reaction with DPNH, the reduction of cytochrome c and the oxidation of DPNH take place stoichiometrically (Table I). The addition of wheat peroxidase to either reaction mixture caused about 20 per cent inhibition and this effect occurred to the same extent in experiments with either peroxidase 556 or 566 (Fig. 5).

TABLE I

Oxidizing Activities of Particles from Wheat Germ

Substrate	DPNH				Succinate	
	Reduction of cytochrome c		Oxidation of DPNH		Reduction of cytochrome c	
	Activity*	%	Activity*	%	Activity*	%
Control system	14.7	100	7.2	100	5.0	100
Addition of antimycin A	4.8	36	2.3	32	0.3	6
Addition of peroxidase	11.8	80	13.0	180	4.0	80

* Activities are expressed as μ moles of reduced cytochrome c or oxidized DPNH per hour per mg. nitrogen of particulate suspension.

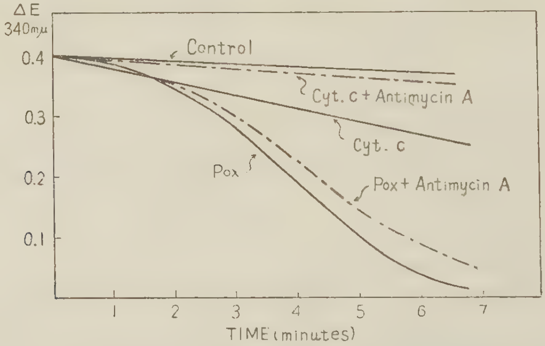


FIG. 6. Oxidation of DPNH by a particulate suspension.

Control reaction mixture contained 0.1 *M* sucrose, 0.05 *M* phosphate buffer, pH 7.4, 1×10^{-4} *M* DPNH and a particulate suspension containing 90 μg. nitrogen. Other reagents were added as indicated in the figure in the same quantities as in Figs. 4 and 5.

Oxidation of DPNH—As illustrated in Fig. 6, DPNH was more slowly oxidized than the control, aerobically in a particulate suspension, unless cytochrome c was added. When a small amount of cytochrome c was added to this mixture, the reaction rate was greatly increased. This reaction was inhibited by antimycin A to the same extent as the reduction of cytochrome c.

Contrary to inhibition produced in the case of cytochrome c reduction, addition of peroxidase to the reaction mixture greatly accelerated DPNH oxidation. The reaction was little affected by antimycin A, and completely inhibited by 10^{-3} *M* cyanide (Fig. 6). Under anaerobic conditions, this effect of the peroxidases on DPNH oxidation was not observed. In the presence of cytochrome c, oxidation of DPNH proceeds stoichiometrically (Fig. 7).

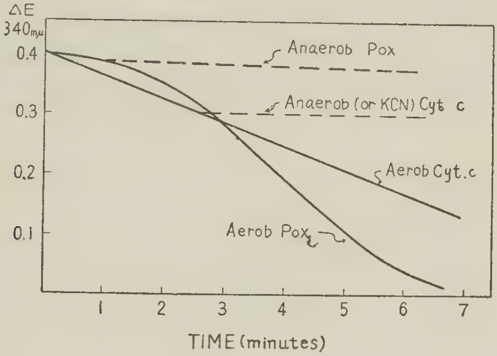


FIG. 7. Oxidation of DPNH in a particulate suspension under various conditions. Experiments under anaerobic conditions were carried out in a Thunberg cuvette. Reagents were added to the reaction mixture as described in Fig. 6.

From these findings it is clear that when peroxidase is added to the mixture, oxidation of DPNH also takes place *via* another system catalyzed by peroxidase.

DISCUSSION

In the present study when either peroxidase 556 or 566 was incubated anaerobically with substrate in a homogenate or particulate suspension, neither the reduced bands of these peroxidases nor that of "cytochrome dh" (*3*, *10*) could be detected. Martin and Morton (*11*) had also failed to observe them. However, because the 'dh' complex appeared only under special conditions such as in a non-streaming oxygen-free medium, further experiments are required to detect "cytochrome dh".

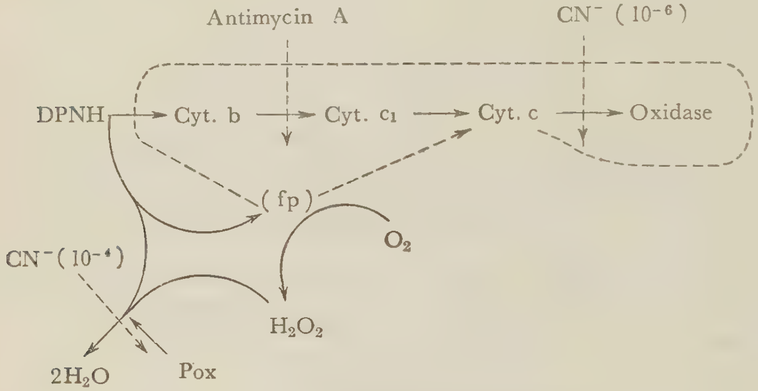
Martin and Morton (*12*) obtained microsomes and mitochondria from wheat root and showed that the DPNH-cytochrome c reductase in mitochondria was 30 per cent inhibited by antimycin A but that of microsome was not inhibited. The particles from wheat germ were not identified as mitochondria in this work, but seemed to correspond to the mitochondria of wheat root, because of their similar metabolic pattern. In the mitochondria, it appears that cytochrome b participates in DPNH oxidation because it is reduced by DPNH anaerobically, and the reaction is inhibited by antimycin A.

In the presence of peroxidase, the oxidation of DPNH and TPNH as well as that of dihydroxymaleic acid (*13*, *14*) has been observed by many workers (*15–19*) to have a specific requirement for manganous ion. Conn *et al.* (*15*) described the DPNH oxidation by an extract of wheat germ containing peroxidase and later found that DPNH was aerobically oxidized by peroxidase in the presence of manganous ion and a suitable phenolic compound (*16*). Stern and Johnston (*19*) obtained particles from wheat embryo which could oxidize DPNH independently of the cytochrome system in the presence of manganous ion.

From the present work, it appears that, the effect of peroxidase on the DPNH oxidation in a particulate suspension is also due to a peroxidatic reaction, because the reaction is completely inhibited by 10^{-3} *M* cyanide or in anaerobic conditions. From available evidence, the scheme shown in Scheme 1 is suggested.

SCHEME 1

Scheme for the particulate electron transporting system in the presence of peroxidase.



(fp) in the figure represents flavoprotein.

It is possible that the hydrogen peroxide necessary for the peroxidatic reaction is produced through aerobic oxidation of the substrate catalysed by a flavin enzyme contained in the particles. In the presence of peroxidase, DPNH as well as cytochrome c is oxidized through a peroxidatic reaction. Consequently the reduction of cytochrome c tends to be inhibited (Fig. 5). However, there is no evidence that the peroxidase is important for cell metabolism.

SUMMARY

1. Evidence is presented that particles from wheat germ possess cytochrome components a, b and c, and their cytochrome system is capable of oxidizing substrates.

2. In the presence of a homogenate or particles of wheat germ, neither peroxidase 556 nor 566 is reduced by any substrate tested under anaerobic conditions, and consequently the presence of "cytochrome dh" was not established.

3. In the presence of peroxidase, the reduction of cytochrome c undergoes approximately 20 per cent inhibition whereas the oxidation of DPNH is greatly accelerated.

4. The oxidation of DPNH by peroxidase in the particulate suspension is completely inhibited by 10^{-3} M cyanide and by anaerobiosis.

5. A possible metabolic scheme for the particulate electron transporting system in the presence of peroxidase is discussed.

The authors are greatly indebted to Prof. K. Okunuki and Dr. B. Hagihara for their kind advice during this work.

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AMINO ACID DECARBOXYLASES OF PROTEUS MORGANII

I. EFFECT OF 8-AZAGUANINE ON THE FORMATION OF HISTIDINE DECARBOXYLASE

By SOHACHI ANDO

(*From the Institute of Food Microbiology, Chiba University, Narashino*)

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Etiological examinations of allergic food poisoning have revealed that *Proteus morganii* is one of the causative bacteria of the poisoning (*1*, *2*). It has also been shown that the allergic symptoms of the poisoning are caused by histamine, which is formed by the action of histidine decarboxylase in the bacteria (*1*, *2*). As described by Gale (*3*), we confirmed that the histidine decarboxylase in this system also is formed through an inducible process. In connection to these findings, authors attempted to analyze the mechanism involved in the induced formation of histidine decarboxylase in *Proteus morganii*. The present paper deals with examinations of cultural conditions required for the induction of the enzyme formation and on the bacterial growth with special reference to the relationship between the syntheses of nucleic acids and enzyme in this organism.

EXPERIMENTALS

Cultural Conditions—*Proteus morganii* was grown in a medium of following composition: $Na_2HPO_4 \cdot 12H_2O$ 18 g., KH_2PO_4 1 g., NH_4Cl 1 g., $MgSO_4 \cdot 7H_2O$ 0.01 g., $FeSO_4 \cdot 7H_2O$ 0.001 g., sodium glutamate 1.7 g., glucose 10 g., cystine 23 mg., niacinamide 12 mg., calcium pantothenate 100 μg., distilled water to make 1 liter, and adjusted to pH 7.2. The cultivation was conducted at 30° for 18 hours.

Enzyme Induction—Bacterial cells harvested from the growth medium followed by washing with saline were suspended in an induction medium to the final concentration of about 1.8 mg. dry weight of cells per ml. unless otherwise stated. The induction medium contained $5 \times 10^{-3}\,M$ L-histidine, an inducer, in place of glutamate in the abovementioned growth medium and adjusted to pH 6.0. Cells were incubated in this medium at 30° for 3 hours. Aliquots were taken out at one hour intervals for the estimation of enzyme activity and nucleic acid content.

Estimation of Enzyme Activity—An aliquot of the induction system was centrifuged, washed with saline, resuspended in a phosphate buffer (pH 6.0) to the concentration of 1.8 mg. dry weight of cells per ml. A mixture of 1.0 ml. of the washed cell suspension and 0.8 ml. of 0.1 M phosphate buffer (pH 6.0) was placed in a main compartment of a Warburg vessel and 0.2 ml. of 0.05 M L-histidine hydrochloride dissolved in the same buffer in a side arm. After equilibration at 30°, histidine was mixed with the cell suspension. The time course of gas evolution was generally linear during the first 15 minutes after mixing and histidine decarboxylase activity estimated during this period. The activity was expressed in terms of μl. CO_2 evolved per hour. Since the rate and total amount of carbon dioxide were found to be unaffected by aerobic and anaerobic conditions, estimation of enzyme activity was performed under the air.

Estimation of Ribonucleic Acid (RNA) and Deoxyribonucleic Acid (DNA)—The extraction of nucleic acids was carried out primarily according to the method of Schneider (*4*). An aliquot was adjusted to pH 3–4 with 1 *N* sulfuric acid, chilled in an ice bath, and centrifuged under chilling. The cells were washed with saline, precipitated at 0° with trichloroacetic acid at a final concentration of 10 per cent, and washed with ethanol and ethanol-ether. RNA and DNA were then extracted from the cells with 5 per cent trichloroacetic acid at 100° for 30 minutes. Separate extraction of RNA and DNA, if necessary, was performed by the method of Schmidt-Thanhauser-Schneider (*4*). RNA was estimated by the orcinol method (*4*) and DNA determined with *p*-nitrophenylhydrazine, as described by Webb and Levy (*5*).

Paper Chromatography and Electrophoresis—Bacterial cells washed with trichloroacetic acid, ethanol and ethanol-ether were hydrolyzed in 1 *N* KOH for 18 hours at 37°. The hydrolysate was approximately neutralized with Amberlite IR-120 (H form) and supernatant was acidified by the addition of formic acid to make the precipitation of DNA and protein fractions complete. Chromatography of mononucleotides contained in the supernatant RNA fraction was run on a Toyo filter paper No. 51 with solvent system of isopropanol-ammonia (*6*). The paperchromatogram thus obtained was further subjected to electrophoresis on another dimension. It was conducted in 0.05 *M* formate buffer (pH 3.1) for 2 hours at about 20 v./cm. after the modified method of Markham and Smith (*6*). Mononucleotide spots thus separated two dimensionally were detected by ultraviolet absorption and the spot of 8-azaguanylic acid on the paper was visualized easily by the characteristic fluorescence emitted under the illumination of ultraviolet light (*7*). The spots of guanylic and 8-azaguanylic acids were eluted with 0.1 *N* HCl and estimated spectrophotometrically by using the extinction coefficient, 12.2 at 255 mμ (*8*).

Bacterial Growth—Bacterial suspension was diluted properly with saline and optical density measured turbidimetrically with an electrophotometer using a green filter. Viable cell counts were carried out by the capillary tube method of Yanagita (*9*).

RESULTS

Formation of Histidine Decarboxylase—As shown in Fig. 1, when *Pr. morganii* was incubated in the induction medium, activity of histidine decarboxylase increased markedly preceded by a short lag and attained maximum after 3 hours, while optical density of the suspension increased almost linearly. The increase in optical density was about 1.6 times during initial 3 hours. In a parallel experiment, viable count in the system was found to increase in proportion to the increase in the optical density.

In *Pr. morganii* the coexistence in induction medium of both glucose and ammonium salt was found to be an essential factor for the induction of the decarboxylase. The depletion of either one or two of these nutrients from the complete induction medium resulted not only in negative formation of the enzyme but in no increase in optical density, as shown in Table I.

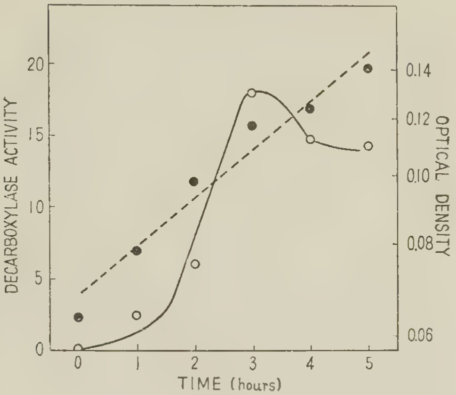

FIG. 1. Formation of histidine decarboxylase and change in optical density in *Proteus morganii*.

Solid and broken lines represent the enzyme activity and the optical density, respectively.

TABLE I

Requirement of Carbon and Nitrogen Sources to the Induced Formation of Histidine Decarboxylase in Proteus morganii

	Induction medium			
	Complete system	Less glucose	Less NH_4Cl	Less glucose and NH_4Cl
Optical density				
0 hr.	0.240	0.260	0.240	0.255
1	0.295	0.255	0.245	0.260
2	0.320	0.255	0.265	0.265
3	0.360	0.255	0.270	0.265
Decarboxylase activity ($\mu l.$ $CO_2/hr.$)	77	0	0	0

It was reported in the induction of lysine decarboxylase (*10*) or histidine decarboxylase (*11*) that carbon and nitrogen sources other than the inducer were required in *Escherichia coli* and that the bacterial cell mass increased twofold during the induction period. Mandelstam (*12*), on the other hand, showed that in *Bacterium cadaveris* the formation of lysine decarboxylase was induced only in the presence of glucose and inducer (lysine) without addition of any other nitrogen sources and that no increase in cell mass occurred. The discrepancy between these observations may be explained by the consideration that *Bact. cadaveris* might be able to utilize free amino acids sufficiently present in the cells for the synthesis of lysine decarboxylase, while in *Pr. morganii* or in *E. coli* the amount of the free amino acids might be insufficient for the synthesis of the enzymes in question.

To clarify this point, an experiment was designed to demonstrate the induced formation of histidine decarboxylase in *Pr. morganii* under a certain experimental conditions. The organism, in this case, was incubated in the abundant exogenous supply of amino acids suppressing cellular growth under the influence of so-called crowding effect. Thus, 0.2 per cent casamino acid in place of ammonium salt was added to the induction medium and cells suspended in it at 10 times higher concentration (18 mg. dry weight of cells per ml.) than that in the preceding experiments. The results obtained from the experiments were rather negative (Table II). When the growth of cells were markedly suppressed by high cellular density, the induction of the enzyme formation also was highly or even completely inhibited independent of the kind of nitrogen sources supplied.

TABLE II

Effect of Cellular Concentration for the Induced Formation of Histidine Decarboxylase in Proteus morganii

Cellular concentration (*mg. dry wt./ml.*)	Nitrogen source in induction medium	Enzyme activity (*μl. CO_2/hr.*)	Optical density 0 hr.	Optical density 3 hrs.
1.8	NH_4Cl (0.1%)	90	0.065	0.125
18	NH Cl (0.1%)	0	0.065	0.093
18	Casamino acid (0.2%)	10	0.065	0.078

Effect of pH on the Formation of Histidine Decarboxylase—Washed cells were incubated in the induction media containing 0.1 *M* phosphate buffer adjusted to pH's ranging from 5 to 7.5. Each culture was centrifuged after 3 hours of incubation, washed with saline under cooling, and enzyme activity estimated at usual way. The decarboxylase activity of the cells incubated at pH 6.0 was arbitrarily taken as 100. Relative values of enzyme activities thus induced at various pH's shown in Fig. 2. It was found from these results that the decarboxylase is induced optimally at around neutrality.

In connection to these observations, it may be of considerable interest to know the pH-activity relationship of the histidine decarboxylase thus induced in *Pr. morganii*. The cells were preincubated in induction medium of pH 6.0 for 3 hours. Aliquots of the preadapted cell suspension were tested for the decarboxylase activities at various pH's using 0.1 *M* phosphate buffer. As shown by broken line in Fig. 2, rather narrow range of pH-activity curve was obtained. Close coincidence of the curve with that for enzyme induction suggests that the process of enzyme induction and the activity of the enzyme induced is dependent upon similar pH values.

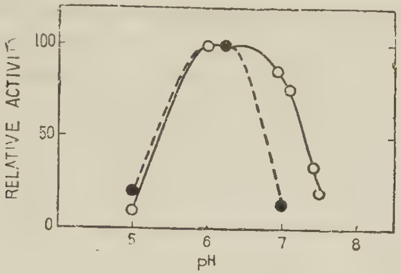

FIG. 1. Effect of pH on the induction and the activity of histidine decarboxylase in *Proteus morganii.*

Ralative values are indicated on an ordinate taking the value observed at pH 6.0 as 100. Solid and broken lines represent the inductive capacity of unadapted cells and enzyme activity of preadapted cells, respectively.

Effect of Concentration of Inducer on the Rate of Formation and on the Activity of Histidine Decarboxylase—Washed unadapted cells were incubated in induction media containing varied concentrations of histidine for 3 hours and the rate of formation of the enzyme determined. In Fig. 3 relative rate of enzyme induction is shown on an ordinate taking the maximum rate observed at the concentration of 0.1 M as 100. From the results shown by a solid line, the concentration of histidine for 50 per cent maximal formation of the enzyme was found to be 6.8×10^{-4} M.

As performed in the preceding pH experiments, the effect of substrate concentration on histidine decarboxylase activity of adapted cells was studied. Cells were preadapted to histidine under optimum conditions and the enzyme activity measured in the presence of varied concentration of histidine. The results are shown by a dotted line in Fig. 3. The "apparent" K_m value for the enzyme activity is shown to be 9.4×10^{-4} M. It is of considerable interest that the dependencies on histidine concentration of the rate of histidine decarboxylase formation and of the activity of the enzyme thus induced seem to be closely related to each other.

Effect of 8-Azaguanine on the Formation of Histidine Decarboxyase—Induced formation of β-galactosidase in *Staphylococcus* was found to be accompanied by no growth of cells during induction period and to be inhibited by 8-azaguanine (*13*). In *Pr. morganii* the induction of histidine decarboxylase formation should occur only when the cellular growth proceeded. Therefore, one is tempted to consider that the effect of 8-azaguanine on the induction mechanism of the enzyme in *Pr. morganii* may differ in some respect from that in *Staphylococcus.*

The cells were incubated for 3 hours in the induction medium in the presence or absence of 10^{-3} M 8-azaguanine, centrifuged followed by washing, and the enzyme activity estimated. It is shown in Table III that the enzyme formation in the presence of antagonist was approximately 20 per cent of the control. Noteworthy is the fact that the viable cell count increases at a similar rate in either case. It was thus found that the induced enzyme formation was inhibited strongly by the antagonist leaving cellular growth unaffected.

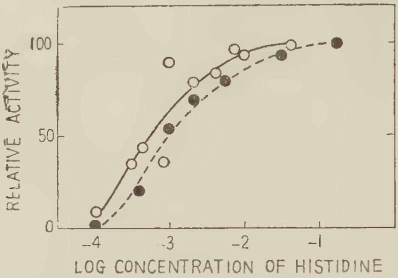

FIG. 3. Effect of histidine concentration on the rate of formation and on the activity of histidine decarboxylase.

Relative values are indicated on an ordinate taking the value observed at 0.1 *M* as 100. Solid and dotted lines represent the rate of formation and the activity of preadapted cells, respectively.

TABLE III

Effect of 8-Azaguanine on the Induced Formation of Histidine Decarboxylase in Proteus morganii

Compound added to induction medium ($10^{-4}M$)		Enzyme activity ($\mu l.\ CO_2/hr.$)	Viable cell count (10^3 cells/ml.)	
			0 hr.	3 hrs.
None		66	1.03	2.01
Guanine	1	97		
Adenine	10	81		
Uracil	10	79		
8-Azaguanine	10	13	0.85	2.00
8-Azaguanine and guanine	10 1	115		
8-Azaguanine and adenine	10 10	180		
8-Azaguanine and uracil	10 10	13		

In the induction of the enzyme formation the presence of either one of 3 bases, guanine (10^{-4} *M*), adenine (10^{-3} *M*) and urcail (10^{-3} *M*), seemed to favor the rate of reaction. The inhibition of the enzyme induction caused by 8-azaguanine was apparently affected by the presence of some of these bases. As indicated in Table III, the addition of guanine or adenine reversed the inhibition and even markedly accelerated the rate of enzyme synthesis, while that of uracil did not show any sign of reversal of the inhibition.

According to Ben-Ishai and Volcani (*14*), when RNA synthesis is inhibited by 2-azaadenine and other base analogues, the protein synthesis also is suppressed. In *Pr. morganii* 8-azaguanine exhibited marked inhibitory activity on the induced formation of histidine decarboxylase leaving cellular growth (cytoplasmic synthesis) unaffected. It is, therefore, considered to be necessary to check the synthesis of nucleic acid under the influence of the antagonist. Thus, the amount of nucleic acids was determined before and after the induction period. Results shown in Table IV indicate that no difference is noted in the rates of syntheses of nucleic acids between treated and untreated cells with the antagonist, while conspicuous difference in the rate of enzyme formation between these two cases is observed.

TABLE IV

Synthesis of Nucleic Acid in the Presence and Absence of 8-Azaguanine during Induction

Compound added to induction medium	RNA (μg./20 ml. culture) 0 hr.	3 hrs.	DNA (μg./20ml. culture) 0 hr.	3 hrs.	Enzyme activity (μl. CO_2/hr.)
Expt. No. 1					
None	380	710	130	200	69
8-Azaguanine 10^{-3} *M*	390	860	140	195	10
Expt. No. 2					
None	295	600	95	315	59
8-Azaguanine 10^{-3} *M*	295	740	135	370	0

Washed cells of *Proteus morganii* were incubated with histidine 5×10^{-3} *M* in the presence and absence of 8-azaguanine 10^{-3} *M* at 30°.

TABLE V

Incorporation of 8-Azaguanine into RNA of Proteus morganii induced in the Presence of 8-Azaguanine

Nucleotide	Amount (μM/ml. eluate)
Guanylic acid	207
8-Azaguanylic acid	54

Separate nucleotides were eluted with 0.1 *N* HCl and estimated spectrophotometrically by using the extinction coefficient 12.2 at 255 mμ.

Since the enzyme induction was found to be inhibited by 8-azaguanine, the incorporation of the antagonist into RNA *de novo* synthesized may be possible. Therefore, the RNA was extracted from the treated cells and the possibility of the presence of 8-azaguanylic acid in the molecule examined. The presence of the mimetic nucleotide in the RNA is evidently shown in Fig. 4. From the estimation of guanylic and 8-azaguanylic acids, it was found that the fraction of guanine residues replaced by 8-azaguanine are 20 per cent (Table V). It was thus disclosed that 8-azaguanylic acid is substituted for guanylic acid amounting 20 per cent of the latter in molar basis.

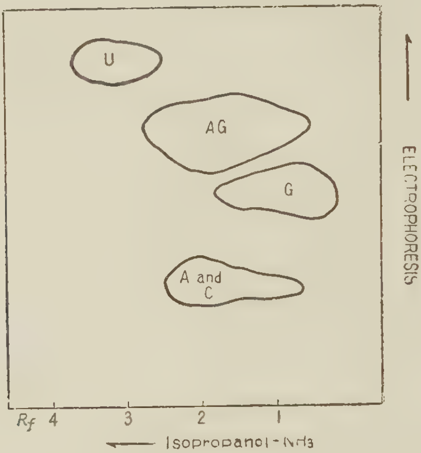

FIG. 4. Separation of nucleotides contained in alkaline hydrolysate of RNA extracted from *Proteus morganii*, incubated in induction medium with the addition of 8-azaguanine (10^{-3} *M*), for 3 hours at 30°.

For paper chromatography and electrophoresis see text. U, AG, G, A, and C represent uridylic, 8-azaguanylic, guanylic, adenylic, and cytidylic acids, respectively.

DISCUSSION

Much work on the induced formation of amino acid decarboxylase has been reported. Nutritional requirement for the induction, however, seem to be rather variable in case by case. Even in the induced formation of a certain amino acid decarboxylase, the nutritional condition favorable to the induction sometimes differs with the organism employed, *e.g.*, the formation of lysine decarboxylase is induced in *Bacterium cadaveris* in the absence of nitrogen source (*12*), while the same enzyme is induced in *Escherichia coli* only in the presence of nitrogen source (*10*). The nutritional requirement in the enzyme induction may be classified into two groups. The one requires the presence of carbon and nitrogen sources plus inducer (CN-type) (*10*, *11*, *15–17*), and the other that of carbon source plus inducer (C-type) (*12*). It has generally been observed that cellular growth proceeds during induction period in the CN-type, while no growth occurs in the C-type. The induced formation of histidine decarboxylase in *Proteus morganii* can naturally be categorized in the

CN-tpye. The difference in the mechnism involved in the induction of enzyme formation in these two types are as yet unsolved. It is, however, highly probable as suggested by Mandelstam (*12*), Halvorson and Spiegelman (*18*, *19*) that the induction of enzyme in the organisms of C-type may depend upon amino acid pool in the cell.

Ben-Ishai and Volcani (*14*) found that the addition of nucleic acid antagonist, such as 2-azaadenine and thiouracil, to bacterial culture resulted in the inhibition of the synthesis of RNA and protein. In *Proteus morganii* the effect of 8-azaguanine on the enzyme induction and on growth seemed to be rater complicated. Although the induced formation of histidine decarboxylase was markedly inhibited by the antagonist, the growth of cells remained unaffected. Moreover, the RNA extracted from the cells treated with the antagonist was found to contain 8-azaguanylic acid in its molecule. These facts suggest that the antagonist may inhibit the synthesis of induced enzyme protein leaving that of structural or constitutive proteins unretarded. Indeed, this was already found to be the case in *Staphylococcus*. Creaser (*13*) reported that the addition of 8-azaguanine inhibited the synthesis of an induced enzyme, β-galactosidase, but not the synthesis of an constitutive enzyme, catalase, in this organism.

SUMMARY

When washed cells of *Proteus morganii* was incubated in an induction medium containing histidine (inducer), glucose and ammonium salt, the induced formation of histidine decarboxylase occurred remarkably accompanied by cellular growth. Cultural conditions under which cellular growth was suppressed seemed to be unfavorable to the enzyme induction.

It is of considerable interest that the pH- and substrate (or inducer) concentration-response relationships obtained for the rate of induced formation and the enzyme activity of histidine decarboxylase coincide quite closely.

Addition of 8-azaguanine into the complete system was found to result in the inhibition of the enzyme induction leaving cellular growth unaffected. In this experiment it was revealed that 8-azaguanine was incorporated into RNA molecule substituting 20 per cent of guanylic acid. The possible mode of action of 8-azaguanine was discussed.

The author wishes to thank Prof. K. Miyaki for his interest and encouragement, and Prof. T. Yanagita and Dr. M. Hayashi for their valuable advice and discussion.

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DENATURATION AND INACTIVATION OF ENZYME PROTEINS

XI. INACTIVATION AND DENATURATION OF GLUTAMIC ACID DEHYDROGENASE BY UREA, AND THE EFFECT OF ITS COENZYME ON THESE PROCESSES

By MINORU INAGAKI

(*From the Department of Biology, Faculty of Science, University of Osaka, Osaka*)

(Received for publication, November 27, 1958)

In the previous papers of this series (*1–6*), it was shown that enzymes such as alcohol, lactic, and triosephosphate dehydrogenases and α-amylases cannot be digested by bacterial proteinase without pretreatment. Using the bacterial proteinase method it was found that the "ratio of inactivation" of the enzymes corresponded to the "ratio of denaturation" during urea-, heat-, and acid treatment. In the preceding papers (*7–8*), it was reported that crystalline fumarase was rapidly digested by bacterial proteinase without pretreatment. The oxidized form of cytochrome c (*9*) which can actively transport electrons and active hexokinase (*10*) were shown to be digested by bacterial proteinase and trypsin, respectively. It is, therefore, considered that there are two groups of enzymes, the one resistant to bacterial proteinase and the other digestible even in the active state. Hence the bacterial proteinase method (*1–6*) can be used only for determination of the "ratio of denaturation" of the former group of enzymes.

On the other hand, it has been shown that some dehydrogenases, such as lactic (*6*, *11*), alcohol (*6*), and triosephosphate (*5*) dehydrogenases, were protected by their coenzymes, the oxidized and reduced form of DPN, from denaturation by urea, *etc.*

Kubo *et al.* reported that glutamic acid dehydrogenase was protected from thermal inactivation by the coenzyme DPN^+ (*12*), and that the enzyme protein dissociates into a number of subunits on dilution (*13*). It is almost certain that the dehydrogenase as well as fumarase, which is readily digested by bacterial proteinase without pretreatment, are usually localized in mitochondria (*14*). The glutamic acid dehydrogenase has such interesting properties as described above. Therefore, we have made an attempt to investigate the inactivation and denaturation of the enzyme protein by urea.

This report presents results on the relation between the "ratio of inactivation" and "ratio of denaturation" of glutamic acid dehydrogenase during urea-treatment. These results are based on the observation that the coenzyme, depending on whether it is in its reduced or oxidized form, either accelerates or retards inactivation and denaturation of the enzyme protein.

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* An outline of this work was presented at the 10th Meeting of the Symposia on Enzyme Chemistry, Japan, at Sapporo, in July 1958.

EXPERIMENTAL

Crystalline Glutamic Acid Dehydrogenase—The enzyme was prepared from pig liver using the method of Kubo *et al.* (*13*). It was recrystallized three times before use.

Crystalline Bacterial Proteinase—A crystalline bacterial proteinase preparation (*15*), "Nagarse", was used, which was supplied by the Nagase & Co., Ltd., Amagasaki.

Diphosphopyridine Nucleotides—The oxidized form of DPN was prepared from baker's yeast according to the method of Okunuki *et al.* (*16*). Its reduced form was prepared enzymatically by the method of Rafter and Colowick (*17*).

Assay of Glutamic Acid Dehydrogenase Activity—The activity was determined by a spectrophotometric method at room temperature. Two assay systems were used. The one contained 133 mM sodium glutamate, 100 μM DPN$^+$, 0.1 to 0.3 ml. of the enzyme solution, and 0.1 *M* phosphate buffer, pH 7.4, in a total volume of 3.0 ml. The other contained 5 mM sodium α-ketoglutarate, 5 mM ammonium chloride, 6.5 μM DPNH, 0.1 to 0.3 ml. of the enzyme solution, and 0.1 *M* phosphate buffer, pH 7.4, in a total volume of 3.0 ml. The enzyme activity is represented here by $10^3 \times \Delta E_{340}$ per minute where ΔE_{340} is the increase or the decrease in optical density of the assay mixture at 340 mμ after the addition of the enzyme solution.

The "ratio of inactivation" was calculated from the following equation: Ratio of inactivation=((Initial activity−Residual activity)/(Initial activity))×100.

Determination of the "Ratio of Denaturation"—After urea-treatment, an aliquot of the reaction mixture containing the enzyme protein was mixed with a solution containing an appropriate concentration of the bacterial proteinase and incubated at 30° for 20 minutes. Then 2.0 ml. of the incubation mixture were added to 0.3 ml. of 4 *M* trichloroacetic acid and kept for about one hour. The resulting suspension was filtered and 1.0 ml. of the filtrate was mixed with 3.0 ml. of 0.55 *M* sodium carbonate and 1.0 ml. of 1/5 Folin-Ciocalteu reagent. The optical density of the mixture was measured at 660 mμ after incubation at 40° for 20 minutes. Samples completely denatured by heating at 90° for 10 minutes with urea were also measured in the same way. The "ratio of denaturation" of glutamic acid dehydrogenase was calculated from the following equation: Ratio of denaturation=[((Folin color of the completely denatured sample)—(Folin color of the sample))/(Folin color of the completely denatured sample)]×100.

RESULTS

Inactivation and Denaturation of Glutamic Acid Dehydrogenase by Urea—It has been shown that the "ratio of inactivation" of various enzymes partially inactivated by urea agreed with the "ratio of denaturation" measured by the bacterial proteinase method (*1-6*). In the present investigation, these two ratios were measured on glutamic acid dehydrogenase incubated with 3.3 *M* urea. Unlike the previous enzymes, the "ratio of inactivation" of the dehydrogenase did not agree with the "ratio of denaturation". Inactivation of the enzyme

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* The following abbreviations are used in the present paper: GADH, glutamic acid dehydrogenase; DPN, DPN$^+$, and DPNH, diphosphopyridine nucleotide, its oxidized form, and reduced form, respectively.

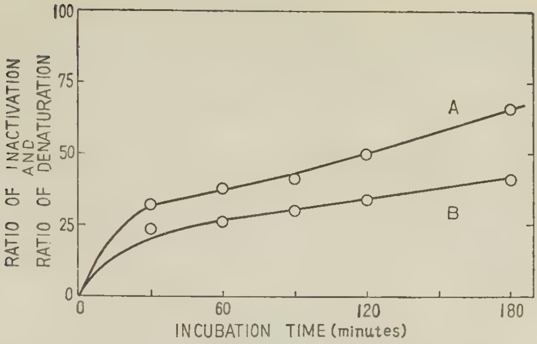

FIG. 1. Time-course of inactivation and denaturation of GADH by urea.

Reaction mixture: GADH, 0.087 per cent; urea, 3.3 *M*; phosphate buffer, 0.1 *M*, pH 7.4. Temperature, 30°.

"Ratio of denaturation" was determined using 2.0 ml. of the mixture and 0.5 ml. of a 0.06 per cent solution of bacterial proteinase by the method described in the text.

GADH activity was measured by reduction of DPN$^+$ using 0.2 ml. of the reaction mixture after diluting one in twelve.

Curve A: Ratio of inactivation.
Curve B: Ratio of denaturation.

always preceded denaturation during the incubation period as shown in Fig. 1.

Inhibitory Action of Urea on Glutamic Acid Dehydrogenase—Since it was thought that the difference between the "ratio of inactivation" and the "ratio of denaturation" might be caused by the inhibitory action of urea on glutamic acid dehydrogenase, the effect of various concentrations of urea on the activity

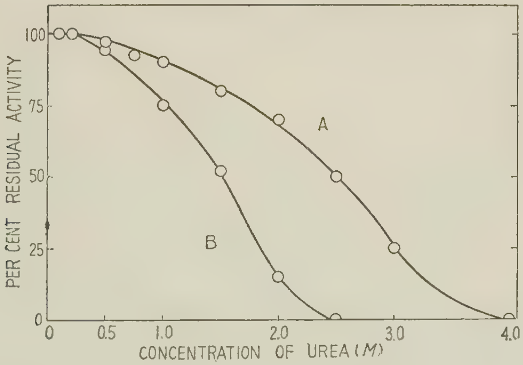

FIG. 2. Inhibition of GADH by various concentrations of urea.

Curve A; measured by reduction of DPN$^+$ in the forward reaction. Curve B; measured by oxidation of DPNH in the reverse reaction. Enzyme activity was measured immediately after the addition of GADH after various concentrations of urea had been added to assay systems. The concentration of GADH was 0.008 per cent for oxidation of DPNH and 0.08 per cent for reduction of DPN$^+$.

was examined. The enzyme was apparently inhibited by the addition of a relatively low concentration of urea as shown in Fig. 2. Urea inhibited the activity of the reverse reaction of the glutamic acid dehydrogenase system, (*i.e.* oxidation of DPNH), more strongly than that of the forward reaction (*i.e.* reduction of DPN$^+$).

In view of this it was of interest to study in detail the inhibitory action of urea on glutamic acid dehydrogenase. Table I shows that the enzyme inhibited by urea regained activity on dilution with buffer. Therefore it seems likely that the inhibitory action of urea on the enzyme is reversible.

TABLE I

Reversibility of Urea Inhibition of GADH

0.5 ml. of 0.03 per cent GADH was incubated with the indicated concentrations of urea or with buffer at 30° for 15 minutes. The total volume was 1.0 ml. 0.1 ml. of the incubation mixture was added to the standard assay system or to an assay system which contained 2 *M* urea and the activity was measured immediately.

Concentration of urea (*M*)		Activity	Per cent residual activity
During pre-incubation	During activity test		
0	0	81	100
2.0	0.067	85	105
0	2.0	16	19.7
2.0	2.067	20	24.7

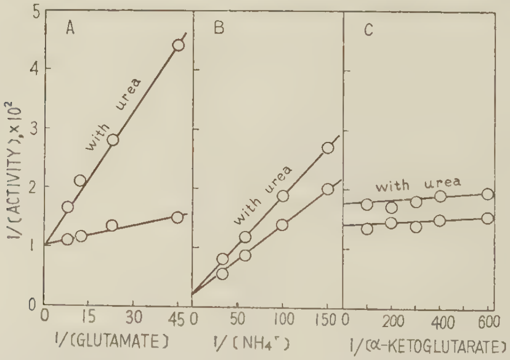

FIG. 3. Influence of substrate concentration on urea inhibition of GADH.

A, GADH, 0.0053 per cent; urea, 2 *M*. B, GADH, 0.00027 per cent; urea, 1 *M*. C, GADH, 0.00053 per cent; urea, 1 *M*.

Activity was measured by the reduction of DPN$^+$ in A, and by the oxidation of DPNH in B and C.

On the other hand, according to a Lineweaver-Burk's plot, urea acts on glutamic acid dehydrogenase as a competitive inhibitor, competing with substrates such as glutamate or the ammonium ion but not with α-ketoglutarate, as shown in Fig. 3.

Effect of Coenzymes and Substrate on the Denaturation of Glutamic Acid Dehydrogenase by Urea—DPN, either in the oxidized or in the reduced form, and substrates have been shown to protect some dehydrogenases from the denaturation by urea and other substances (*5*, *6*, *11*). Therefore, the effect of DPN and substrate on the inactivation of glutamic acid dehydrogenase by urea was examined. DPN^+ and glutamate had a protective action as shown in Fig. 4.

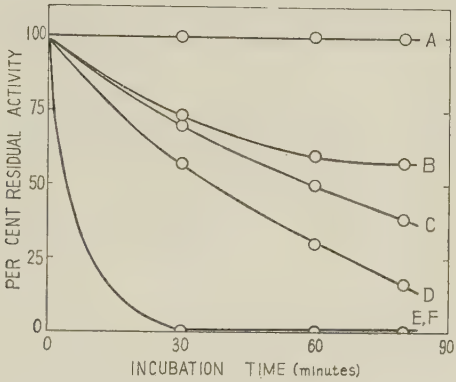

FIG. 4. Effect of substrate and coenzyme on inactivation of GADH by urea.

Reaction mixtures: GADH, 0.42 per cent; urea, 3.6 *M*: addition at concentrations described below. Total volume, 3.6 ml. pH, 7.4. Temperature, 30°.

0.5 ml. of the reaction mixture was added to 12 ml. of phosphate buffer, 0.1 *M*, pH 7.4, at suitable intervals and 0.1 ml. of the diluted solution was used to test the activity. Curve A: No additions. Curve B: Urea and glutamate, 0.37 *M*. Curve C: Urea and DPN^+ 500 μM. Curve D: Urea only. Curve E: Urea and DPNH, 500 μM. Curve F: Urea, glutamate, 0.37 *M* and DPN^+, 500 μM.

On the contrary, reduced coenzyme, DPNH, did not protect the enzyme, but accelerated its inactivation. This was an unexpected result. Since the preparation of DPNH was relatively crude it was thought that an impurity might accelerate the inactivation of the enzyme by urea. However, this possibility was excluded by the following experiment. The enzyme preincubated with a mixture of DPN^+ and glutamate, both of which had separately a protective action, was incubated with urea The rate of inactivation of the enzyme was as rapid as that of enzyme treated with DPNH and urea. It is, therefore, reasonable to conclude that the acceleration of inactivation of glutamic acid dehydrogenase by urea is due to the action of DPNH itself.

The effect of DPNH on denaturation of this enzyme by urea was also examined. The results are summarized in Table II. DPNH accelerates not only inactivation but also denaturation of glutamic acid dehydrogenase by urea.

TABLE II

Effect of Coenzymes on Denaturation and Inactivation of GADH by Urea

Reaction mixture: GADH, 0.04 per cent; urea, 3.8 *M*; additions, 0.4 mg. per ml.; 0.1 *M* phosphate buffer, pH 7.4. Total volume, 2.5 ml. Temperature, 30°. Incubation period, 30 minutes. In the control, buffer solution was added instead of urea and additions.

Ratio of denaturation was determined using 2.0 ml. of the mixture and 0.3 ml. of 0.15 per cent bacterial proteinase. Enzyme activity was measured by the rate of oxidation of DPNH, using 0.2 ml. of the mixture diluted one in twenty with buffer.

Additions	Urea treatment	Activity	Per cent inactivation	Folin color*	Per cent denaturation
None	−	84	—	—	—
None	+	55	35.5	0.044	19.5
DPN^+	−	73	—	—	—
DPN^+	+	69	5.5	0.033	14.5
DPNH	−	78	—	—	—
DPNH	+	11	85.9	0.131	55.6

* Folin color of completely denatured GADH was 0.266.

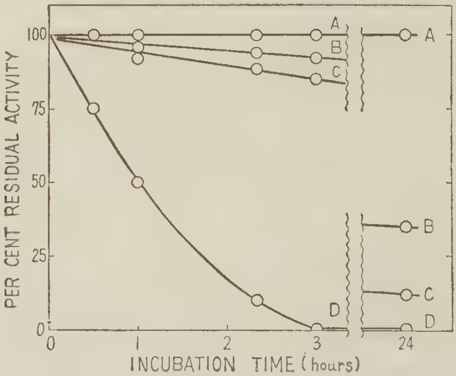

FIG. 5. Effect of coenzymes on action of bacterial proteinase towards GADH.

Reaction mixtures: GADH, 0.017 per cent; proteinase, 0.08 per cent; coenzymes, 0.25 mg. Total volume, 3.0 ml. Temperature, 32°. 0.1 ml. of the reaction mixture was used to assay the activity.

Curve A: No additions. Curve B: Proteinase and DPN^+.
Curve C: Proteinase. Curve D: Proteinase and DPNH.

Effect of Bacterial Proteinase on Glutamic Acid Dehydrogenase—When glutamic acid dehydrogenase was incubated with relatively high concentration of bacterial proteinase for long period, its activity decreased considerably. This inactivation was depressed by addition of DPN^+, but was markedly accelerated by the addition of DPNH as shown in Fig. 5. This shows clearly that the structure of glutamic acid dehydrogenase protein is readily deformed by combination with DPNH, thus becoming more susceptible to attack by proteinase.

DISCUSSION

Though glutamic acid dehydrogenase was inactivated and denatured by a relatively high concentration of urea, the "ratio of denaturation" of this enzyme, unlike that of enzymes reported in previous papers, did not correspond to the "ratio of inactivation". In these experiments the assay system for measuring the activity of the enzyme and the system for digestion of denatured enzyme protein were inevitably contaminated with urea used as the denaturing agent. It is necessary to consider whether the dehydrogenase and bacterial proteinase used for digestion of denatured protein may have been directly inhibited by urea. The dehydrogenase was inhibited by a relatively low concentration of urea (over 0.5 *M* final concentration) but the inhibition was reversible and disappeared on dilution. Therefore, it is impossible that in the experimental conditions used for assay of activity, containing 0.22 *M* urea, direct inhibition of the dehydrogenase raised the value for the "ratio of inactivation". On the other hand, the value for the "ratio of denaturation" might have been affected by the inhibition of bacterial proteinase by urea. However this was not so, since digestion of casein or completely denatured glutamic acid dehydrogenase by proteinase was not influenced by the presence of 4 *M* urea. It is conceivable therefore that the discrepancy between the "ratio of inactivation" and the "ratio of denaturation" is not due to the inhibitory action of urea on the dehydrogenase and proteinase, but to a specific structure of the enzyme protein, which was strongly inactivated even by slight denaturation.

Inactivation and denaturation of glutamic acid dehydrogenase by urea, like that of lactic, alcohol, and triosephosphate dehydrogenases, was protected by the addition of glutamate, the ammonium ion, or DPN^+. However, DPNH did not protect the enzyme but accelerated its inactivation and denaturation by urea. In its native state the enzyme was very slowly attacked by the bacterial proteinase. In this case DPN^+ protected the enzyme. On the contrary, DPNH accelerated the digestion of the enzyme protein by proteinase. It has been reported (*1–6*) that enzyme proteins, such as alcohol, lactic, and triosephosphate dehydrogenases, α-amylase from microorganisms, and catalase, cannot in their native state be attacked by the bacterial proteinase but that if they are denatured they are readily attacked. This phenomenon has been explained by postulating that in their native state the enzyme proteins have a special secondary structure, but when they are deformed during denaturation, so become readily digestible by the proteinase. Therefore, from the instability and the digestibility of glutamic acid dehydrogenase in combination with DPNH, it is suggested that the structure of the protein of glutamic acid dehydrogenase readily becomes deformed by DPNH.

It is interesting that there is an appreciable difference in the effects of the reduced and oxidized forms of the coenzyme on the stability of the enzyme protein. A similar phenomenon has been observed with heme proteins. The reduced form of cytochrome c is less susceptible to proteinase than the oxidized form (*9*). Several proteins related to hemoglobin are susceptible to pancreatin, their susceptibility decreasing in the following order (*18*): Globin > methohemoglobin > oxyhemoglobin > reduced hemoglobin. It has also been reported that oxyhemoglobin differs from reduced hemoglobin in X-ary pattern (*19*). From these facts, it is reasonable to conclude that the structure of the glutamic acid dehydrogenase protein differs in the presence of the reduced or oxidized forms of the coenzyme.

SUMMARY

Inactivation and denaturation of glutamic acid dehydrogenase prepared from pig liver by urea were investigated. Unlike enzymes such as α-amylase, catalase and other dehydrogenases reported in the previous papers, the "ratio of inactivation" of this dehydrogenase does not correspond to the "ratio of denaturation" at any stage of the urea treatment.

Glutamic acid dehydrogenase was reversibly inhibited by a relatively low concentration of urea. The urea competes with the substrates.

DPN^+ and glutamate protect the enzyme from denaturation by urea and attack by bacterial proteinase. On the contrary, DPNH accelerates denaturation by urea and digestion by the proteinase.

From these results it was suggested that the configuration of this enzyme protein is changed depending on whether it is combined with DPN^+ or DPNH.

The author wishes to express his gratitude to Professor K. Okunuki for his guidance, and would like to thank his colleagues Messrs. K. Iwasaki (Nihon Nosan Kogyo Co., Ltd., Yokohama) and T. Masada of this laboratory for helpful discussion during the work.

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SPECTROSCOPIC STUDIES ON THE PHYCOBILIN PIGMENTS OBTAINED FROM BLUE-GREEN AND RED ALGAE*

By AKIHIKO HATTORI AND YOSHIHIKO FUJITA

(*From the Institute of Applied Microbiology, University of Tokyo, Tokyo*)

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As reported in a previous paper (*1*), three phycobilin pigments, *i.e.*, phycocyanin, phycoerythrin and allophycocyanin, have been obtained in highly purified states from a blue-green alga, *Tolypothrix tenuis.* Isolation and purification of the pigments were successfully achieved by a combined use of positive adsorption with tricalcium phosphate and fractional precipitation with ammonium sulfate. By applying the same method to other species of algae (blue-green and red), we obtained equally good results which revealed some new facts pertaining the identity or difference in spectroscopic properties of phycobilin pigments contained in different algae. In the present paper, the spectroscopic characteristics of these pigments obtained from various sources will be described. It will also be shown that allophycocyanin, which has thus far received only a few reference in the literature, has a wide distribution in both blue-green and red algae.

METHODS

Algal Materials—The algae used as material were either those collected from their natural habitat or those grown in pure cultures in the laboratory. Red algae**, *Porphyra tenera*, *Polysiphonia urceolata*, *Grateloupia sp.* and *Carpopeltis sp.* were collected from the sea shore at Aburatsubo, on the Pacific coast. The fresh water alga *Aphanothece sacrum* (Suizen-ji Nori) was obtained in dried state from the market. Laboratory cultures were made of the blue-green algae *Anabaena variabilis***, *Nostoc muscrum**** (using Medium C of Myers *et al.*, (*2*)) and *Tolypothrix tenuis* (using Medium II of Ukai *et al.*, (*3*)) by the method described in a previous paper (*3*).

Isolation of Pigments—The algal material was macerated with a Waring blendor and the homogenate was centrifuged to remove cell fragments. When necessary, the particulate fractions containing chlorophylls and carotenoids were removed by ultracentrifugation (100,000×g, 60 minutes).

First, phycoerythrin in the supernatant solution was separated from phycocyanin and allophycocyanin by precipitation with ammonium sulfate at 30 per cent saturation. Further separation and purification were conducted by combining adsorption with tricalcium phosphate-gel and precipitation with ammonium sulfate, as described in the previous paper (*1*).

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* This work was presented at the Annual Meeting of the Botanical Society of Japan held in Fukuoka on Oct. 25, 1958.

** The red algae were identified by Dr. M. Arasaki, Faculty of Agriculture, University of Tokyo.

*** These strains were obtained from the University of Texas by the courtesy of Dr. J. Myers.

Spectrophotometry—The absorption spectra of the pigments were determined at pH 5.8 (*M*/10 –*M*/20 phosphate buffer), with a Beckman Spectrophotometer Model DK 2.

Estimation of Pigment Content—The pigment content in algal cells was computed from the spectrophotometric readings made with the homogenates of algae, using for each phycobilin pigment values of molar extinction coefficients estimated with highly purified samples obtained from *Tolypothrix tenuis* (phycocyanin, phycoerythrin and allophycocyanin) and *Porphyra tenera* (phycoerythrin).

RESULTS AND DISCUSSION

The absorption spectra of the highly purified samples of the three phycobilin pigments obtained from *Tolypothrix tenuis* and *Porphyra tenera* are

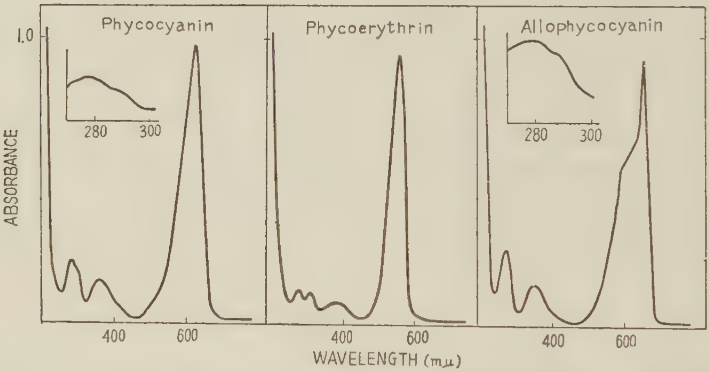

FIG. 1. Absorption spectra of phycobilin pigments obtained from *Tolypothrix tenuis.*

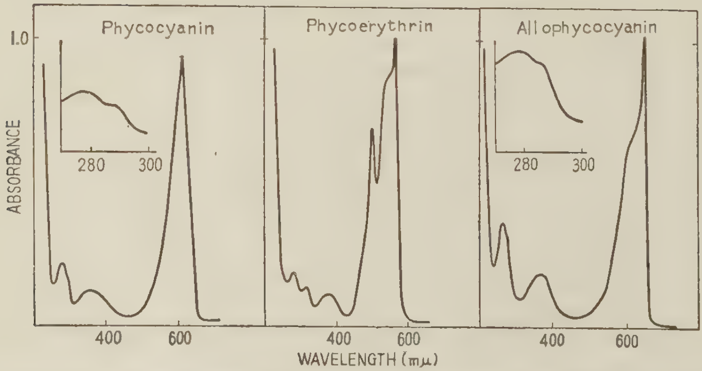

FIG. 2. Absorption spectra of phycobilin pigments obtained from *Porphyra tenera.*

shown in Figs. 1 and 2, respectively. Corresponding data for the pigments from all algal species investigated are summarized in Table I (see also Fig. 4). It will be seen from these results that phycocyanin and allophycocyanin from red algae are spectroscopically identical with those of blue-green algae, so far

TABLE I

Absorption Maxima of Phycobilin Pigments Obtained from Various Algae

Red algae

Algae	Phycobilin pigment	State1)	Absorption maxima ($m\mu$)
Porphyra tenera	Phycocyanin	crys.	280, 285, 360, 615
	Phycoerythrin	crys.	280, 310, 370, 500, 545, 565
	Allophycocyanin	am.	280, 285, 360, 600, 650
Gratelcupia sp.	Phycocyanin	am.	280, 285, 360, 615
	Phycoerythrin	am.	280, 310, 370, 500, 545, 565
	Allophycocyanin	am.	280, 285, 360, 600, 650
Polysiphonia urceolata	Phycocyanin	am.	280, 285, 360, 615
	Phycoerythrin	am.	280, 310, 370, 500, 545, 565
	Allophycocyanin2)		
Carpopeltis sp.	Phycocyanin2)		
	Phycoerythrin	am.	280, 310, 370, 500, 545, 565
	Allophycocyanin2)		

Blue-green algae

Algae	Phycobilin pigment	State1)	Absorption maxima ($m\mu$)
Tolypothrix tenuis	Phycocyanin	crys.	280, 285, 360, 615
	Phycoerythrin	crys.	280, 310, 370, 565
	Allophycocyanin	crys.	280, 285, 360, 600, 650
Aphanotece sacrum	Phycocyanin	am.	280, 285, 360, 615
	Phycoerythrin	am.	280, 310, 370, 565
	Allophycocyanin	am.	280, 285, 360, 600, 650
Anabaena variabilis	Phycocyanin	am.	280, 285, 360, 615
	Phycoerythrin2)		
	Allophycocyanin	am.	280, 285, 360, 600, 650
Nostoc muscrum	Phycocyanin	am.	280, 285, 360, 615
	Phycoerythrin	am.	280, 310, 370, 565
	Allophycocyanin	am.	280, 285, 360, 600, 650

1) Abbreviations used: crys., crystalline; am., amorphous.
2) No attempt was made of the separation of the pigment.

as the algal species studied are concerned*. It may be pointed out in this connection that the earlier claim for the occurrence of two different forms, namely C-phycocyanin in blue-green, and R-phycocyanin in red algae, was erroneous, having most probably been derived from observations made with insufficiently purified samples of the pigment. In Fig. 3 are compared the spectra obtained with phycocyanin samples (from *Porphyra tenera*) at two different stages of purification. The spectrum obtained with the less purified sample was similar to that reported for the so-called R-phycocyanin, showing an additional peak at 550 mμ which was lacking in the sufficiently purified sample**. The peak at 550 mμ was conceivably due to a contamination of phycoerythrin in this sample as well as in the so-called R-phycocyanin.

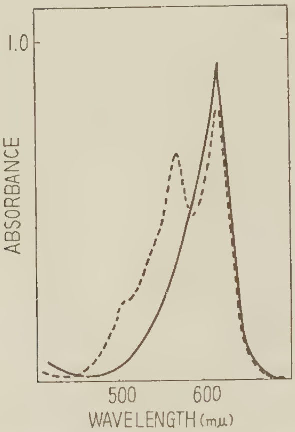

FIG. 3. Absorption spectra of phycocyanin-preparations of different degree of purity prepared from *Porphyra tenera*. Solid line: the highly purified sample; dotted line: the sample contaminated with phycoerythrin.

Several common features were found in the spectra of phycocyanin and allophycocyanin, which—irrespective of their sources—shared the property of showing distinct absorptions of shoulders at 280, 285 and 360 mμ. Among these, the shoulder at 285 mμ deserves an attention because it has not hitherto been recorded in the literature. In contrast to the apparent identity of phycocyanin and allophycocyanin obtained from different algal species, a considerable variety in spectroscopic nature was found in the phycoerythrin obtained from different algae. As may be seen from the data presented in Figs. 1, 2, 4, and Table I, all the phycoerythrin-samples tested showed absorption bands at 565, 370, 310* and 280 mμ; however, some samples showed two additional bands at 545 and 500 mμ, while others were lacking in these. Moreover, the relative intensities of the bands at 565, 545, and 500 mμ were so different from sample to sample that the shape of the whole absorption spectrum varied considerably according to the source of the pigment (Fig. 4). Difference in the properties of phycoerythrin-samples obtained from different sources also manifested itself in their sedimentational behaviours. For example, the sedimentation constant ($s_{20,w}$ at pH 7.2) obtained for the sample from *Tolypothrix tenuis* (*1*) was 10.2, while that for the sample from *Porphyra tenera* was 12.7. The question as to whether the difference in spectroscopic properties of phycoerythrin samples of different origins is due to the presence of different forms of prosthetic groups in the pigment protein, or to the different mode of combination of one and the same prosthetic group with the protein moiety is left open to further investigations.

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* The identity of phycocyanin in *Porphyra tenera* and *Tolypothrix tenuis* has also been confirmed by sedimentation studies, which gave the same value of $s_{20,w}$ (6.1 at pH 7.2) in both cases.

** A single peaked spectrum (in the region from 500 to 700 mμ) of phycocyanin has also been reported by Fujiwara (*4*) and French *et al.* (*5*) who isolated the pigment from *Porphyra tenera* and *P. naiadum*, respectively.

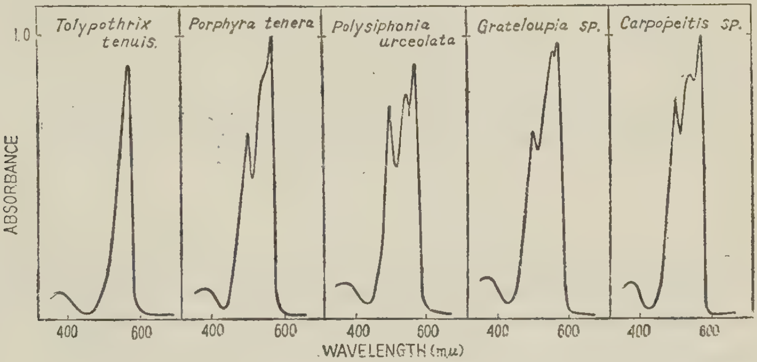

FIG. 4. Absorption spectra of phycoerthrin obtained from various algae.

Data showing the relative content of phycobilin pigments in various algal species are presented in Table II. In conformity with the widespread notion, phycoerythrin forms the major phycobilin pigment in Rhodophyceae, but contrary to the general belief, the same pigment is also predominant in most of the blue-green algae investigated. An interesting case of *Tolypothrix tenuis*, in which the relative levels of phycoerythrin and phycocyanin were subjected to a significant variation according to the wave length of the light applied during the culture (see the brief explanation in Table II) will be dealt with in detail in our subsequent paper. Worthy of notice is the consistent occurrence of allophycocyanin, which has hitherto been considered to be a rather rare component of phycobilin pigments in algae. As may be seen from Table II, its content in red algae often exceeds that of phycocyanin, and attains, in most of the blue-green algae, a level as high as 50 per cent of that of phycocyanin.

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* The peak at 310 mμ, which has been overlooked by many workers in the past, was recently noticed by Haxo *et al.* (*6*) who studied the phycoerthrin from the red alga *Porphyridium cruentum*.

TABLE II

Relative Content of Phycobilin Pigments in Various Algae

Algae	Phycoerythrin	Phycocyanin	Allophycocyanin
Tolypothrix tenuis (C-cells[1])	0.05	1.00	0.42
Tolypothrix tenuis (E-cells[2])	1.54	1.00	0.59
Anabaena variabilis	—	1.00	0.52
Nostoc muscrum	1.20	1.00	0.51
Aphanothece sacrum	1.14	1.00	0.47
Porphyra tenera	1.00	0.13	0.19
Polysiphonia urceolata	1.00	0.10	0.13
Grateloupia sp.	1.00	0.15	0.12
Carpopeltis sp.	1.00	—	0.19

1) Cells grown under red light of wave lengths longer than 600 mμ.
2) Cells grown under blue light of wave lengths shorter than 535 mμ.

SUMMARY

1. Three kinds of phycobilin pigments (phycocyanin, phycoerythrin and allophycocyanin) were isolated and purified from various algae including 4 species of Rhodophyceae and 4 species of Cyanophyceae. Spectroscopic data characterizing the purified pigments were presented, and compared with those reported by earlier investigators.

2. While phycocyanin and allophycocyanin obtained from different algal species were practically identical in their spectroscopic properties, the properties of phycoerythrins varied widely according to their sources. The difference was observed not only with algae of different classes, but also with the algae belonging to the same class (Rhodophyceae). The common spectroscopic feature of phycoerythrins from different sources was the absorption bands at 565, 545, 500, 370, 310 and 280 mμ, the relative intensity of which varied, however, widely from sample to sample.

3. Allophycocyanin was found to be far widely distributed among blue-green and red algae than it has been thought heretofore. It was found to share with phycocyanin the property of showing absorptions or shoulders at 280, 285 and 360 mμ.

4. Relative content of the three phycobilin pigments were determined with different species of algae. As has been generally thought, phycoerythrin forms the major phycobilin pigment in Rhodophyceae, but contrary to the widespread notion, the same pigment was found to be also predominant in most of the blue-green algae investigated.

The authors wish to express their grateful thanks to Profs. H. Tamiya, A. Watanabe and A. Takamiya, University of Tokyo, for their critisisms and encouragements during this work, and for the help in preparation of this paper.

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The Journal of Biochemistry, Vol. 46, No. 7, 1959

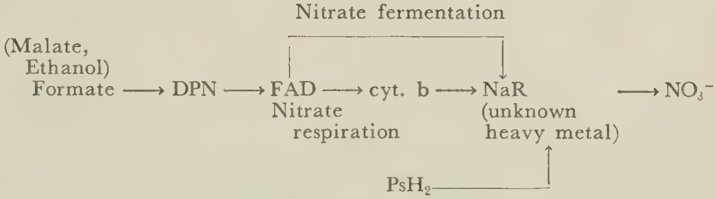
THE SOLUBLE AND CYTOCHROME-LACKING NITRATE-REDUCING SYSTEM IN GERMINATING COTYLEDONS OF BEAN SEED EMBRYOS, VIGNA SESQUIPEDALIS*

By KAZUCHIYO OHMACHI, SHIGEHIKO TANIGUCHI
AND FUJIO EGAMI

(*From the Department of Chemistry, Faculty of Science, Nagoya University, Nagoya*)

(Received for publication, December 10, 1958)

Egami *et al.* found in bean cotyledon macerate a soluble DPN**-linked nitrate reductase which was characterized as a sulfhydryl metalloenzyme (*2*). It has been speculated by these authors that two pathways of electron transfer to nitrate, both functioning in a non-assimilative way***, might coexist in the cotyledon of the younger germinating bean seed embryo, as illustrated by the following scheme:



Cytochrome b, the predominant component of cytochromes of cotyledon tissue in the early stage of germination**** is involved in the electron transport sequence of "nitrate respiration" type (*3*), whereas this is not the case in the system of the so-called "nitrate fermentation" type (*2*). The simultaneous occurrence of both pathways speculated in the previous paper (*2*) for the cotyledon seemed to be probable, in view of the analogous occurrence of two kinds of carbohydrate metabolism in the cotyledon tissue: aerobiosis (respiration) occurring on tissue surfaces and anaerobiosis (fermentation) occurring in the bulk of thick tissue, corresponding to the difference in morphological situations in respect to the availability of oxygen*.

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* Summary of this work was included in the report presented at the International Symposium on Enzyme Chemistry held in Tokyo, Japan, 1957 (*1*).

** The following abbreviations are used: nitrate reductase, NaR; diphosphopyridine nucleotide, DPN; reduced forms of DPN and phenosafranine, DPNH and PsH_2; flavin adenine dinucleotide, FAD; cytochrome, cyt.

*** The non-assimilative nature of nitrate reduction occurring in cotyledons is evidenced by the fact previously reported that the nitrite formed from nitrate is not further reduced in this organ which has, in general, a nature of a "dissimilative" organ.

**** It has been found that the germinating tissue of cotyledon has a cytochrome pattern of "a, b_1" type in earlier stages (2 day-old), and that of "a, b, c" type in later stages (6 day-old).

Owing to the low contents both of nitrate reductase** and cytochrome b together with the strictly sterile procedure necessary to avoid the contamination of nitrate-reducing bacteria, it has been difficult to separate these two enzymes in a preparative manner.

The present paper will show that the separation can be achieved successfully by a centrifugal fractionation procedure, and it will be demonstrated that a cytochrome-lacking pathway of nitrate fermentation type is predominantly functioning in the germinating cotyledons of bean seed embryos.

EXPERIMENTALS

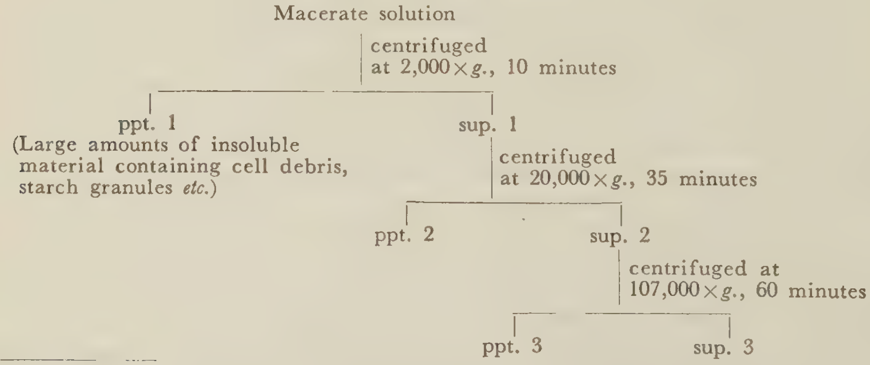
The sterile procedure from sowing the seeds to preparation of the enzyme solution (cotyledon macerate) and assay conditions were previously described (*2*). The centrifugal fractionation of macerate solutions of 2 day-old cotyledon was carried out also under sterile and low temperature conditions (below 5°).

Ultracentrifugation was carried out using a preparative Spinco centrifuge (Model L). The sterility of each fraction was confirmed by bacterial growing test. Details of examination of cytochrome spectra and assay method of nitrate reductase activity were also previously described (*2*).

RESULTS

The whole procedure of centrifugal fractionation of cotyledon macerate solution is shown by the following flow-sheet:

Flow-sheet of Centrifugal Fractionation of Macerate Solution from 2 Day-old Vigana Cotyledons


Macerate solution
centrifuged at 2,000 × *g*., 10 minutes
ppt. 1
(Large amounts of insoluble material containing cell debris, starch granules *etc.*)
sup. 1
centrifuged at 20,000 × *g*., 35 minutes
ppt. 2
sup. 2
centrifuged at 107,000 × *g*., 60 minutes
ppt. 3
sup. 3

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* Personal communication from Dr. Y. Oota of the Department of Biology.

** The activity of cotyledon nitrate reductase was far less (about one-hundredth) than that of microbes, *e.g.*, *Cl. welchii* which was found to possess a similar nitrate reductase system of nitrate fermentation type (Dr. J. Koyama in the Institute for Infectious Diseases, University of Tokyo, unpublished data, reported at the Annual Meeting of the Japanese Biochemical Society, 1955.).

Two precipitated fractions (ppt. 2 and ppt. 3), both of reddish brown color, were resuspended in the original volume of 0.05 *M* phosphate buffer, pH 7.5. Data showing the distribution of nitrate reductase activity and cytochrome b are summarized in Table I.

When assayed using formate with DPN as an electron-transferring system, 50 per cent of the nitrate reductase activity of macerates was extracted in the sup. 3 fraction in a soluble form. However, when assayed using reduced phenosafranine as an electron donor which probably donates its electrons more directly, much portion of the original activity was recovered in the sup. 3 fraction. In the latter case, the sup. 3 fraction of yellow color was found to contain almost all portion of the enzyme in a soluble form, resulting in a 3- to 4-fold increase in the specific activity* compared with that of macerate solution.

TABLE I

Distribution in Various Fractions of Nitrate Reductase Activities and Cytochrome b after Centrifugal Fractionation of Cotyledon Macerate Solution from 2 Day-old Vigna Embryos

Electron donor Fraction	Nitrate reductase units1)		Intensity2) of α-band (565 mμ) of reduced cyt. b
	Formate, DPN	Reduced phenosafranine	
Macerate	22	49	
sup. 1	22	50	
sup. 2	14	47	+
ppt. 2	5	11	⧻
sup. 3	11	58	−
ppt. 3	2	8	+

Reaction mixture:

In formate-DPN system: each fraction, 3.0 ml. (corresponding to 3 pairs of homogenated cotyledons) containing 0.15 m moles phosphate buffer, pH 7.5; formate, 50 μ moles; DPN (70 per cent), 1.6 μ moles; added KNO_3, 10 μ moles.

In reduced phenosafranine system: formate and DPN were replaced by reduced phenosafranine (with Pd and H_2 gas), 7.5 μ moles.

Total volume in each test solution, 6.0 ml. in Thunberg tube. Temperature, 30°.

1) Enzyme unit was expressed as μ moles of nitrite produced in one hour.

2) According to the observations made with an Ocular Spectroscope, this b-type cytochrome was found to have its reduced band (produced by dithionite) at 565 mμ (α) and 525 mμ (β), respectively.

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* Specific activity was expressed as units/mg. N. Concerning the definition of enzyme unit, see the note below Table I.

The spectroscopic examination revealed that the fraction sup. 3 was apparently free from cytochrome b as well as from any other cytochromes, and cytochrome b was concentrated almost all in the precipitated fraction, especially in the ppt. 2 fraction.

In the case assayed using formate with DPN, the decrease of the activity in the soluble form compared with that of the original activity is possibly due to the stepwise removal of formic dehydrogenase of particulate form during the centrifugal fractionation*. In this case, the nitrate reductase activity recovered in sup. 3 in the soluble form is probably limited by the lower level of formic dehydrogenase activity of the soluble form detectable in sup. 3.

In the case assayed using reduced phenosafranine, however, the terminal part of the nitrate-reducing system: nitrate reductase itself seems to be all in the soluble form apart from cytochrome b.

As previously reported, the activity of macerate, regardless of the kind of electron donors used, was not appreciably inhibited by the inhibitors acting upon the cytochrome system, *i.e.*, 2-heptyl-4-hydroxyquinoline-*N*-oxide (HOQNO) or 2-hydroxy-3-(2-methyloctyl)-1,4-naphthoquinone (SN 5949).

Summarizing all, we may infer that in the cotyledon tissue, not only nitrate reductase itself but also the whole system of the electron transfer from formate *via* DPN to nitrate seems to be predominantly functioning in the soluble form apart from cytochrome b.

DISCUSSION

It has been reported that some microorganisms having a cytochrome pattern of "a, b_1" type (*e.g.*, *E. coli*) have a particulate nitrate-reducing system involving cytochrome b_1 which was classified as "nitrate respiration" type (*3*, *4*). In these cases, b-type cytochrome was often found to be present even in a highly purified preparation of nitrate reductase as demonstrated in those obtained from *E. coli* (unpublished data, 1958) and also *Pseudomonad* (*5*).

In the cotyledon tissue, owing to the cytochrome-lacking and soluble nature decisively confirmed here together with the low potential nature of the cotyledon system, which has been revealed in our earlier studies, the previous proposal by Egami *et al.* (*2*) of the concept of nitrate fermentation appears to be more appropriate to characterize this non-assimilative metabolism. Thus, in the cotyledon, a nitrate-reducing system of "nitrate fermentation" type, and not of "nitrate respiration" type, may be predominantly functioning as one of the possible energy-yielding mechanisms as previously illustrated (*2*).

The heavy metal component of the nitrate reductase of this type remains to be clarified.

SUMMARY

Further evidence was presented by the centrifugal separation of nitrate

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* According to the recent work by Mr. K. Horiuchi (1958, unpublished data), a major part of formic dehydrogenase activity of young germinating cotyledons was found to reside in the similar particulate fraction.

reductase activity and cytochrome b to show decisively that the electron transfer for nitrate reduction in the cotyledon of vigna bean in earlier stages of germination occurs predominantly in the following manner:

$$\begin{tikzcd}
\text{(Malate, Ethanol) Formate} \arrow[r] & \text{DPN} \arrow[r] & \text{FAD} \arrow[r] & \text{NaR (unknown heavy metal)} \arrow[r] & \text{NO}_3^- \\
& & \text{PsH}_2 \arrow[ur] & &
\end{tikzcd}$$

Although the cotyledon system is of non-assimilative nature, this system cannot be classified in the " nitrate respiration " type, since the above-mentioned pathway is a soluble system and involves no cytochrome. The physiological significance of the nitrate reduction occurring in the cotyledon system seems to be that of the energy-yielding " nitrate fermentation ", the concept of which was previously proposed by Egami *et al.*

The authors wish to express their sincere thanks to Prof. Takeshi Mori in the Department of Biology who kindly helped our spectroscopic observation of cytochromes.

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The Journal of Biochemistry, Vol. 46, No. 7, 1959

STUDIES ON CYTOCHROME A

III. EFFECT OF SYNTHETIC DETERGENTS UPON THE ACTIVITY OF CYTOCHROME A

By TAKASHI YONETANI

(*From the Department of Biology, Faculty of Science,*
University of Osaka, Osaka)

(Received for publication, December 12, 1958)

As reported in the previous communications of this series (*1*, *2*), cholic acid was used as a reagent for extraction or solubilization of cytochrome a from beef heart muscle. After fractionation with ammonium sulfate the cytochrome a preparation contained no other cytochrome components and had almost all the spectrophotometric properties specific to cytochrome oxidase; that is, cytochrome a can combine with oxygen and with specific inhibitors such as cyanide, carbon monoxide and nitric oxide, and an oxygenated form of cytochrome a can be converted to its oxidized form in the presence of cytochrome c. On the other hand, the purified preparation showed only weak cytochrome oxidase activity. Since cholic acid is known to inhibit its activity (*3*, *4*), it seems to be very important to check the inhibition of cytochrome a by cholic acid and to examine whether the activity lost during the purification procedure can be recovered by means of other type of detergents. In a preliminary report (*5*), it was shown that Emasol 4130, a synthetic non-ionic detergent, effectively reactivated a purified cytochrome a. Similar reactivation of particulate cytochrome oxidase was also reported by Wainio and Aronoff (*6*). In the present communication more detailed results are given of these experiments (*5*) and a comparison is made on some properties of cytochrome a in a solution of cholic acid and of Emasol 4130.

EXPERIMENTAL

Materials—Cytochrome a was prepared according to the method described in the previous communication (*1*) except that it was not treated with alumina C_γ gel. Crystalline cytochrome c was prepared from beef heart by the method of Hagihara *et al.* (*7*). The designation of the fractions of cytochrome a is the same as in the 1st paper of the series (*1*). The various types of detergents used in this experiment were commercial products and their compositions and the names of the companies in which they were prepared are shown in Tables I and II. Other materials used were the same as previously described (*1*, *2*).

Methods—The sample was suitably diluted with 0.1 *M* phosphate buffer containing 1 per cent cholic acid at pH 7.4 and reduced with sodium dithionite. Then the concentration of cytochrome a was determined from the difference in light absorption at 605 mμ and 630 mμ, using the value of 16.5 $\text{mM}^{-1}\times\text{cm}^{-1}$. which was calculated from the results given in the 1st paper (*1*). In the preliminary experiments (*5*), we adopted the value reported by Wainio (*8*) but this method did not sufficiently reduce the error caused by turbidity. The cytochrome oxidase activity was assayed manometrically in a Warburg apparatus. The reaction mixture contained 0.2 ml. of 2×10^{-4} *M* cytochrome c, 0.2 ml. of cytochrome a suitably diluted with 0.1 *M* phosphate buffer, pH 7.4, 1.2 ml. of 10^{-1} *M* buffer of pH 7.4, and 0.2 ml. of 10 per cent detergent in the main compartment, 0.2 ml. of 10^{-1} *M* hydroquinone in the side arm, and 0.1 ml. of 20 per cent potassium hydroxide in the center well. The total volume was adjusted to 2.1 ml. with water and the final concentration of cholic acid in the reaction mixture was 0.06 per cent unless otherwise described. The reaction was begun by addition of hydroquinone and the oxygen uptake was measured at 5 minutes intervals at 30°. The buffers used were Tris-HCl and phosphate at pH 7.4. The activity was about 1.5 times larger in Tris-HCl than in phosphate. The concentration of protein was determined by the biuret-reaction according to the method of Gornall *et al.* (*9*), bovine blood albumin serving as the standard. The specific activity was expressed as μM oxygen taken up per mg. of protein per minute and the turnover number was μM oxygen taken up per μ mole of cytochrome a per minute at 30°.

RESULTS

I) Effect of Various Types of Synthetic Detergents upon the Activity of Purified Cytochrome a—Fraction S_3 was chosen as the purified preparation of cytochrome a and the effect of various types of synthetic detergents upon its activity was examined manometrically in Tris-HCl buffer of pH 7.4. Table I shows that the activity increased 2 to 4 times on addition of all the types of non-ionic detergents examined, while, as illustrated in Table II, cationic and anionic detergents, except for Quatamin-86, were very inhibitory. Polyalcohols acted like non-ionic detergents but had little effect. Among the non-ionic detergents examined, the polyoxyethylene sorbitan-fatty acid ester type showed a high reactivating and solubilizing effect. Emasol 4130 was chosen as the most suitable detergent for the preparation of cytochrome a in the following experiments because the detergent is very soluble even in the presence of much salt.

II) Effect of the Concentration of Emasol 4130—Fig. 1 shows the effect of the concentration of Emasol 4130 upon the activity and solubilization of cytochrome a. In the presence of 0.06 per cent cholic acid at pH 7.4, a solution of cytochrome a was completely clear at a concentration between 0.01 and 5 per cent of the detergent. The highest activity was observed at a concentration between 0.1 and 5 per cent. From the above results, a concentration of 1 per cent of Emasol 4130 was chosen as a suitable one for giving a preparation of cytochrome a with good activity.

III) Effect of Emasol 4130 upon the Activity of Various Fractions of Cytochrome a during Its Purification—As reported in the preliminary paper (*5*), the activity in 0.06 per cent cholic acid was inhibited by the addition of Emasol 4130 at earlier stages of purification, but the ratio of inhibition gradually decreased as purification proceeded. After the complete removal of cytochrome b, the

TABLE I

Effect of Non-ionic Detergents upon the Activity of Purified Cytochrome a

Agent	Composition	H.L.B.1)	Manufacturer2)	Activity3)	Relative activity (%)
				15	100
Emulgen-120	Polyoxyethylene-lauryl ether	15.5	K	37	247
Emulgen-420	Polyoxyethylene-oleayl ether	13.5	K	39	261
Neugen ET-140	Polyethyleneglycol-oleayl ether	14.0	D	25	167
Neugen ET-170	Polyethyleneglycol-lauryl ether	17.0	D	47	313
Emulgen-910	Polyoxyethylene-alkylphenol ether	10.0	K	23	154
Neugen EA-142	Polyethyleneglycol-octylphenol ether	14.0	D	19	126
Neugen EA-143	Polyethyleneglycol-dodecylphenol ether	14.0	D	28	187
Neugen EA-170	Polyethyleneglycol-nonylphenol ether	17.0	D	37	246
Emanon-1112	Polyethyleneglycol-monolaurate	13.2	K	36	240
Emanon-3135	Polyethyleneglycol-monostearate	14.3	K	63	420
Emanon-4115	Polyethyleneglycol-monooleate	12.3	K	41	274
Neugen ES-160	Polyethyleneglycol-oleate ester	15.1	D	41	274
Neugen 10-SA	Polyethyleneglycol-stearate ester	17.0	D	55	367
Solgen-70	Sorbitan monopalmitate	7.0	D	61	407
Tween-20	Polyoxyethylene-sorbitan-monolaurate	16.7	A	62	414
Tween-80	Polyoxyethylene-sorbitan-monooleate	15.0	A	52	347
Emasol-1112	Polyoxyethylene-sorbitan-monolaurate	13.2	K	44	293
Emasol-1130	Polyoxyethylene-sorbitan-monolaurate	18.6	K	58	387
Emasol-3130	Polyoxyethylene-sorbitan-monostearate	16.4	K	53	353
Emasol-4130	Polyoxyethylene-sorbitan-monooleate	16.5	K	62	414
Nonion ST-221			N	44	293
Amilagin	Polyoxyethylene alkylamine ether	3	D	44	293
Aminon	Polyoxyethylene fattyamine		K	42	280

1) Hydrophilic and lyophilic balance.

2) K, Kao Soap Co., Ltd.; D, Daiichi Kogyo Seiyaku Co., Ltd.; A, Atlas Powder Co., Ltd.; N, Nihon Yushi K.K.

3) Oxygen uptake (μl.) per 10 minutes.

preparation began to be reactivated by Emasol 4130 and the ratio of reactivation increased with purification. The specific activity did not decrease as it did the turnover number (Table III).

IV) Comparison of the Properties of Cytochrome a in a Solution of Cholate or Emasol 4130—a) Absorption Spectrum: No difference was observed between the absorption spectra of cytochrome a in a solution of 1 per cent cholate and in that of 1 per cent of Emasol 4130.

TABLE II

Effect of Ionic Detergents and Polyalcohols upon the Activity of Purified Cytochrome a

Type	Agent and composition		Manufacturer1)	Activity2)	Relative activity (%)
				15	100
Anion	Neogen T	Alkylbenzene-sulfonate	D	2	13
	Neogen TS		D	2	13
	Sodium dodecylsulfonate			1	7
	Demol N	Sodium dinaphthalene-sulfonate-HCHO condensate	K	0	0
	Pelex OT	Sodium alkylsulfosuccinate	K	1	7
Cation	Quatamin-24	Alkyltrimethylammonium choloride	K	5	33
	Quatamin-86		K	12	80
	Polyvinyl alcohol			15	100
	Polyethyleneglycol			26	173
	Propyleneglycol			12	80

1), 2) see Table I.

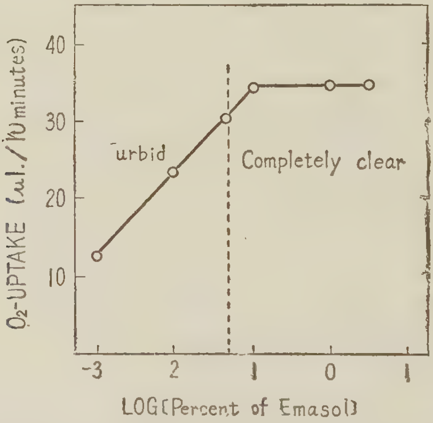

FIG. 1. Effect of concentration of Emasol 4130 upon the activity and solubilization of cytochrome a.

Fraction S_4 was used and the experimental conditions were described in the text.

b) *Substrate Specificity*: The substrate specificity of a particulate cytochrome oxidase preparation has already been reported by Okunuki (*10*). Almost similar results were obtained with Fractions S_2 and S_4 in a solution of 0.2 per cent cholic acid or 1 per cent Emasol 4130. Among the substrates used, hydroquinone and *p*-phenylenediamine were the most suitable for activity.

c) Optimum pH: Since cytochrome a in a solution of Emasol 4130 was quite clear even at a strongly acidic pH, the effect of pH upon the activity was examined. When hydroquinone was used as substrate, the pH optimum was between 7 and 8. At acidic pH's the activity decreased rapidly and at above pH 8 autoxidizability of the substrate increased. Cytochrome a itself was labile at above pH 9.

TABLE III

Effect of Emasol 4130 upon the Activity of Various Fractions during the Purification of Cytochrome a from Beef Heart

Preparation was started with 800 g. of heart muscle. Emasol 4130 was added to the reaction mixture at a final concentration of 1 per cent.

Fraction	Total volume (*ml.*)	Total protein (*mg.*)	Cytochrome a		Specific activity (O_2 μM/*mg. protein/min.*)		Turnover number (μMO_2 /μM *Cyt. a/min.*)		Total activity (%)	
			(μM)	10^{-3} μM *per mg. of protein*	− Emasol	+ Emasol	− Emasol	+ Emasol	− Emasol	+ Emasol
Particulate prep.	400	30,400			0.13	0.10			100	82
S_1	400	6,400	15.3	2.4	0.25	0.14	100.4	55.8	42	24
S_2	90	5,220	10.6	2.0	0.15	0.11	70.4	55.8	20	16
S_3	55	440	2.7	6.2	0.07	0.27	10.8	44.5	0.8	3.2
S_4	22	352	2.4	6.9	0.01	0.21	0.9	29.8	0.1	1.7

TABLE IV

Effect of Carbon monoxide upon the Activity of Various Fractions

The activity was measured after suitable dilution. The final concentration of cholic acid was 0.2 per cent and that of Emasol 4130 was 1 per cent.

Fraction		Activity (μ*l.* O_2/*10 min.*)		Inhibition (%)
		95%N_2+5%O_2	95%CO+5%O_2	
Particulate preparation	light	45	45	0
	dark	45	29	35
S_2 (in cholate)	light	29	29	0
	dark	29	18	38
(in Emasol)	light	21	21	0
	dark	21	14	37
S_4 (in cholate)	light	60	60	0
	dark	60	36	40
(in Emasol)	light	80	80	0
	dark	80	20	75

d) Inhibitors: Table IV shows the effect of carbon monoxide on the activity of various fractions of cytochrome a. Almost the same inhibition

TABLE V

Effect of Inhibitors on the Activity of Fraction S_4 in 1 per cent Emasol 4130

Inhibitor	Final concentration (*M*)	Activity (*μl.* O_2/*10 min.*)	Inhibition (%)
None		50	0
CN^-	10^{-5}	20	60
	10^{-4}	0	100
N_3^-	10^{-3}	14	72
Fe $(CN)_6^{-4}$	10^{-2}	25	50
Thiourea	10^{-2}	50	0
8-Oxyquinoline	10^{-2}	50	0
EDTA[1]	10^{-2}	50	0
$\alpha\alpha'$-Dipyridyl	10^{-2}	50	0
o-Phenanthroline	10^{-2}	50	0
DDC[2]	10^{-2}	—[2]	0[2]

1) Ethylenediamine tetraacetic acid.

2) Diethyldithiocarbamate. The activity was measured with ascorbate in place of hydroquinone as substrate.

was observed in all preparations except Fraction S_4, in which the inhibition was duplicated in Emasol 4130. The inhibition was completely reversed by light irradiation.

Table V shows the effect of other inhibitors on the activity of Fraction S_4 in Emasol 4130. The same effect of inhibitors was observed in other fractions and in both detergents.

e) *Stability*: Fractions S_2 and S_4 were very stable in 1 per cent cholic acid and in 1 per cent Emasol 4130 and there was scarcely any loss of activity after storage for a month at 4°.

DISCUSSION

Cytochrome a purified by means of cholic acid and by fractionation with ammonium sulfate can transfer electrons from cytochrome c to oxygen, but the activity is very weak in the final stage of the purification. Since as reported in the lst paper (*1*) the activity diminished as purification proceeded, it was attempted to prevent the loss of activity by addition of cytochrome c and 10 per cent sucrose. These additions gave about 20–30 per cent reactivation in each fraction, but did not maintain the activity at a constant level. It was then examined the effect of detergents other than cholic acid, because cholic acid is an anionic natural detergent and inhibition by various anions has been reported by Miyajima (*11*). Wainio and Aronoff (*6*) reported that natural detergents such as cholate, deoxycholate and digitonin, and synthetic non-ionic detergents such as Tween had an activating effect on preparations of particulate cytochrome oxidase, but the activity could only be extracted from the particle by means of the natural agents. In a similar manner, the effect of various detergents was examined on the activity of purified cytochrome a. As illustrated in Tables I and II, all types of synthetic non-ionic detergents reactivate the purified preparation, but ionic-detergents are very inhibitory. To clarify the effect of non-ionic detergents, Emasol 4130 was chosen as most suitable for our purposes and its effect on various fractions of cytochrome a was studied. Though it is very difficult to obtain a constant value because of variations in stability of cytochrome b or its complex in cholic acid solution, a typical result is shown in Table III. By addition of Emasol 4130 to the reaction system, we succeeded in maintaining the turnover number of a purified preparation at 1/2–1/4 of that of the first extract. As can be seen, however, reactivation by Emasol 4130 does not appear at the earlier stages of purification, but only after the removal of the cytochrome b component from the preparation. In the later stages of purification, rapid decrease in the turnover number is observed in cholic acid, but this decrease is slow in Emasol 4130. From the above results, it seems that the loss of activity during purification procedure is due to at least two factors; the first is the removal from the preparation of a lipid or lipid-like cofactor associated with the cytochrome b complex and the second is the inhibition by cholic acid. The latter is overcome by the addition of Emasol 4130 to the reaction system. Thus our purified preparation, Fraction S_3, retains a turnover number of 45, and this corresponds to about 1/6 of that given by Chance (*12*) for a particulate preparation.

Other properties of cytochrome a, that is, the absorption spectrum, substrate specificity, pH optimum, and behavior towards inhibitors, were also compared in a solution of cholic acid and of Emasol 4130. Almost the same properties were observed in either solutions. Thus, Emasol 4130 is very advantageous for use in the investigation of the mechanism of the cytochrome oxidase reaction.

Extraction of cytochrome a from a particulate preparation with Emasol 4130 was also examined, but was not successful. It is well known that cytochrome a is closely bound to mitochondria by lipid and cytochrome a itself can be extracted by detergents such as cholate. This means that cholic acid is useful for solubilization of the lipid associated with cytochrome a. In our experience, however, in the presence of ammonium sulfate cholic acid has another effect in degrading the fragment of cytochrome a from a macro-molecule to a simplified protein. Thus, in the use of Emasol 4130 for extraction of cytochrome a many points remain to be resolved.

SUMMARY

1. By the addition of non-ionic detergents, the activity of purified cytochrome a is restored to about 1/4 of its initial activity. Ionic-detergents are inhibitory.

2. During the purification of various fractions, inhibitory effect of cholate is completely reversed by Emasol 4130 (a non-ionic detergent).

3. Cytochrome a has the same properties in a solution of cholic acid as in Emasol 4130.

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ACTIONS OF RADIATIONS ON ENZYMES

II. BIOCHEMICAL EFFECTS CAUSED BY IRRADIATIONS OF γ-RAY ON DEHYDROGENASES

By SHOZO TANAKA, HIROYUKI HATANO AND SHIGETAKE GANNO

(*From the Department of Chemistry, Faculty of Science,*
Kyoto University, Kyoto)

(Received for publication, December 19, 1958)

It has been well established that even relatively low doses of several ionizing radiations often cause inactivation of enzymes which have sulfhydryl radicals as active groups in their dilute aqueous solutions (*1*, *2*). Such inhibition of enzyme activities has been attributed to oxidation of the sulfhydryl groups into the disulfide groups brought about by the irradiated products of water molecules. The enzymes thus inactivated are proved to be capable of being reactivated by reduction of the disulfide groups with thiol compounds such as reduced glutathione and cysteine. In the case of enzymes whose active group has been shown to be something other than sulfhydryl, however, an irreversible inactivation has always been brought about by larger doses of radiation such as causing denaturation of protein (*3*, *4*). The non-recoverable inhibition of this kind is, therefore, presumed to be due to some chemical change or changes occurring in a group which is different from SH in the molecule of enzyme protein or of coenzyme. Also in these cases the changes occurring in structures of protein or of coenzyme may be assumed to be caused by the irradiated products of water.

This paper is concerned with the experimental results of the effects of γ-ray irradiation on crystalline alcohol dehydrogenase (ADH) from baker's yeast[1] as well as purified glutamic dehydrogenase (GDH) from the beef liver[2].

EXPERIMENTALS

Preparation of Alcohol Dehydrogenase from Bakers's Yeast—Alcohol dehydrogenase was prepared from baker's yeast[3] by Racker's procedure (*5*) with some modifications[4]: 1 pound of the fresh cake of yeast was crumbled and kneaded with 200 ml. of purified toluene for an hour at about 38°. About 190 ml. of 1 *M* disodium phosphate was

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[1] We are particularly grateful to Mr. O. Itasaka and Mr. T. Okamoto for their technical assistance.

[2] A part in this work has been reported at the IInd Symposium on Atomic Energy in Japan, Tokyo, 1958.

[3] The so-called Oriental yeast was chosen as the material for these experiments.

[4] Iwatsubo, M., personal communication.

added to the paste thus obtained until its pH became 7.4. The mixture was left for an hour at about 38°, and then kept at about 0° overnight. A spoonful of Hyflo-Super-Cel was added to the mixture, and after thorough mixing, the suspension was filtered through a layer of Hyflo-Super-Cel of 0.5 cm. depth. A clear, yellowish filtrate obtained was 334 ml. in volume, and showed a specific activity of 6,900 units of the enzyme. The extract was heated for 5 minutes at about 50° and was filtered. To 338 ml. of the filtrate 115 g. of ammonium sulphate were added and the mixture was allowed to stand for 30 minutes at 0°. The precipitate produced was collected by centrifugation at 8,000 r.p.m. for 15 minutes at 0°. The sediment was dissolved again in 20 ml. of cold water and 4 g. of ammonium sulphate were added to the solution. The resulting precipitate was filtered off and discarded. On adding gradually a saturated solution of ammonium sulphate to the filtrate, crystalline enzyme began to sediment out at 0.35 saturation of ammonium sulphate, and the addition of ammonium sulphate in small portions was continued for about half an hour until a 0.6 saturation was reached. The mixture was left for about 10 hours at 0°. The crystallized enzyme collected from 137 ml. of the mixture by centrifugation (at 8,000 r.p.m. for 20 minutes) was again dissolved in about 6 ml. of redistilled water and recrystallized in the same way.

Preparation of Glutamic Dehydrogenase from Beef Liver—Glutamic dehydrogenase was isolated from fresh beef liver according to the methods by Olson (*6*), Strecker (*7*) with some modifications (*8*). With 3 l. of redistilled water, 1 kg. of the fresh beef liver was homogenized by the help of a mixer. To the homogenate 150 g. of sodium sulphate were added and the solution was stirred for 5 minutes at about 55°, pH 6.4. A spoonful of Hyflo-Super-Cel was added to the solution which was then filtered through a layer of Hyflo-Super-Cel (1 cm. in thickness) on filtere paper (Toyo Roshi No. 1). The residue was extracted again with 0.1 *M* phosphate buffer solution at pH 6.4 and the extract was brought together with the above filtrate. The total volume of the filtrate amounted to 2,500 ml. To the solution 375 g. of sodium sulphate were added and the precipitate produced was collected by filtration. The precipitate was dissolved in 250 ml. of 0.1 *M* phosphate buffer solution at pH 6.4 and heated for 5 minutes at 55°. To 325 ml. of the solution thus obtained 27.6 g. sodium sulphate were added. After removing the precipitate, 11.5 g. of sodium sulphate was again added to the filtrate. The precipitate was dissolved in 83 ml. of 0.1 *M* phosphate buffer solution containing 2×10^{-3} *M* of ethylendiamine tetraacetate, and 7.8 g. of sodium sulphate were again added to the solution. The precipitate produced was collected by centrifugation at 10,000 r.p.m. for 15 minutes at 0°. The sediment was dissolved again in 34 ml. of 0.1 *M* phosphate buffer. On standing overnight at 0° in a refrigerater, glutamic dehydrogenase was crystallized out of this solution which had 1,070 units of activity of this enzyme per mg. of enzyme protein.

Preparation of Diphosphopyridine Nucleotide from Fresh Yeast—Diphosphopyridine nucleotide (DPN) used in the experiments was prepared from fresh baker's yeast by Okunuki's method (*9*) and purified by the procedure reported by Kornberg and Pricer (*10*). 10 kg. of fresh cake of baker's yeast were crumbled and the coenzymes were extracted with 8 l. of hot water for 10 minutes at 80°. After cooling as quickly as possible, the pH of the extract was adjusted to 2.0 with 12 *N* hydrochloric acid and the mixture was filtered through Hyflo-Super-Cel. The extract was passed through a column (50 cm. in length and 30 cm². in cross section) of Duolite C-10 (H form, 60 to 100 mesh) at a rate of 3 l. per hour. Elution of the adsorbed matter was carried out with 1 *N* aqueous ammonia at a flow rate of a liter per hour. Neutral fractions were collected consecutively and adjusted to pH 7.0 with 6 *N* aqueous ammonia. The solution was again passed through a column of 14 cm. × 30 cm^2. of 200 to 400 mesh of Dowex 1×2 (formate form), at a rate of 10 ml. per minute. After washing with 100 ml. of water, the adsorbate was eluted with 0.1 N formic acid at a flow rate of 10 ml. per minute. With each consecutive of the eluate, the content of DPN was estimated by the alcohol dehydrogenase method. The fractions which were proved to contain more than 5 mg. of DPN per ml. were gathered and adjusted to pH 2.0 with 6 N nitric acid. To the solution, 9 volumes of 99 per cent ethanol were added and the mixture was left overnight at 0°. The precipitate produced was collected by centrifugation and washed twice with 99 per cent ethanol and again with absolute ether, and then dried in vacuum over calcium chloride. The yield of DPN thus obtained amounted to 0.8–1.0 g. and its purity was proved to be about 70 per cent.

Measurement of Dehydrogenase Activity—The activity of dehydrogenases was determined from the amount of reduced diphosphopyridine nucleotide, DPNH, by measuring the intensity of its light absorption at 340 mμ at 25° (*5, 7, 8, 11*). The determination of the activity was carried out as follows; a mixture was made of 10–20 μg. of alcohol dehydrogenase in 0.1 ml., 0.1 ml. of 3 M ethanol solution, 0.1 mg. of DPN in 0.1 ml., 0.5 ml. of 0.06 M pyrophosphate buffer pH 8.5 and 50–90 μg. of glutamic dehydrogenase in 0.1 ml., 0.1 ml of 0.33 M sodium glutamate, 0.5 mg. of DPN in 0.5 ml. and 2.3 ml. of 0.2 M potassium phosphate buffer pH 7.6, and the final volume of the solution was made up to 3.0 ml. and after a definite time interval the light absorption at 340 mμ was measured in the usual way. The increment in density between the 15th and 30th second readings after addition of the substrate, times 4 was taken as the enzyme activity per minute.

γ-Irradiation Technique—Procedures used were essentially the same as described in a previous paper (*2*); for γ-irradiation 50 c. of Co^{60} in Toshiba RIT-I Teletherapy Unit* were used as a source of γ-ray. Enzyme preparations contained in a glass tube of 0.8 cm. in diameter and 5 cm. in length, were exposed to doses of γ-rays at 0° in an ice bath during irradiations. For measurements of radiation doses of γ-rays, the chemical dosimetry by ferrous sulphate (*12*) was employed. The solutions of 10^{-3} M ferrous ammonium sulphate in 0.8 N H_2SO_4 containing 10^{-3} M of sodium chloride, were irradiated by γ-rays. The optical absorption of the ferric sulphate at 305 mμ was estimated using a Beckman's Model DU spectrophotometer. In the measurement of the dose rate, an ionic yield (G-value) of 15.5 mol. ferric ion per 100 eV. was used for calculation.

RESULTS AND DISCUSSION

As shown in Table I, alcohol dehydrogenase of the yeast was inhibited easily by rather lower doses of γ-ray.

Glutamic dehydrogenase was also inactivated by γ-irradiations. On plotting the decrease of its activity against the dose of γ-rays applied, the curve shown in Fig. 1 was obtained.

From this curve which shows the relation between the inactivation of enzymes and the dose of γ-rays, the protection of the active enzyme by the already inactivated enzyme molecules against oxidation was also noticed as shown in the previous works (*2, 3*). The inhibition of enzyme activity by given doses of γ-rays was brought about independently of the amount of enzymes in the irradiated solutions in so far as the concentration of enzyme solutions was proportional to the activity of enzymes. Deviation from this rule was observed only in extremely dilute solutions, in which relatively stronger inhibition took place, as may be seen from the results shown in Fig. 2. The above-mentioned relationship between the enzyme concentration in aqueous solution and the doses of radiation applied had been emphasized by Dale *(3)* as an evidence for an indirect action of radiations upon enzymes.

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* The equipment in the University Hospital of Kyoto was used: The authors express their gratitude to Prof. T. Fukuda, Kyoto University, for his permission to use it.

TABLE I

Effect of γ-Rays on the Activities of Yeast Alcohol Dehydrogenase

γ-Ray dose (*r*)	Enzyme activity[1] (*unit*)	Inhibition (%)
0	54	—
400	50	7·42
800	36	33·2
6,000	25	53·7
10,000	14	74·2

[1] The solution containing 1 μg. in 0.2 ml. of ADH of 50,000 units per mg. of protein was used to measure the activity of enzyme.

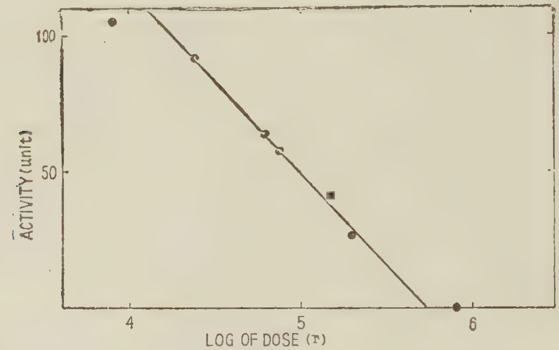

FIG. 1. Inhibition of the activity of liver glutamic dehydrogenase by γ-rays. (GDH, 90 μg./0.1 ml.)

Apoenzymes of yeast alcohol dehydrogenase as well as of liver glutamic dehydrogenase were observed to be inactivated by γ-rays in dilute aqueous solutions. The common coenzyme for both enzymes, DPN, was proved to be more resistant against γ-ray than apoenzymes, but its inactivation occurred when larger doses of γ-ray were applied. The effect of irradiation on DPN as it was reflected in the change of ultraviolet spectrum is shown in Fig. 3. A decrease in optical density at 260 mμ should be attributed to the breakdown of DPN molecule in the adenine moiety. The inactivation of enzyme was found to be proportional to the change in optical density at 260 mμ of DPN as shown in Table II.

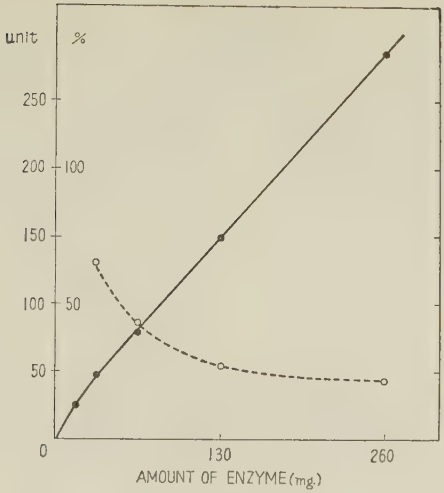

FIG. 2. Activity (—) and sensitivity towards γ-rays (---) of liver glutamic dehydrogenase as measured at different concentration of the enzyme. (γ-ray, 50,000 r)

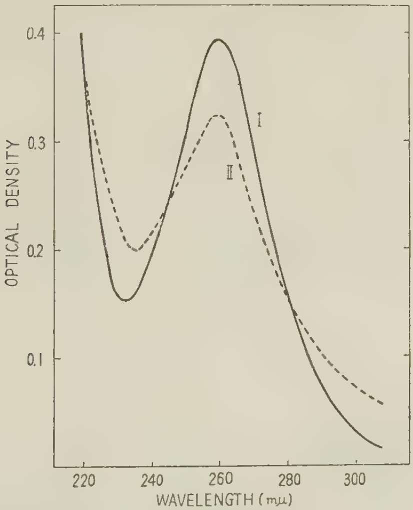

FIG. 3. Ultraviolet absorption spectra of DPN before (I) and after (II) γ-irradiation. (DPN 50 μg./ml., γ-ray 50,000 r, light path 0.1 cm.)

The apoenzyme of glutamic dehydrogenase was mixed with DPN in several different ratios, and the effect of γ-ray upon the systems was investigated. The experimental results obtained are given in Table III. Although there was no quantitatively definite relationship to be found, it was fairly clearly established that DPN exerted a protecting effect upon the apoenzyme against irradiation.

TABLE II

Changes in Ultraviolet Absorption Spectra and in the Activity of DPN[1]

Dose (r)	Decrease of absorbance at 260 mμ (%)	Activity[2] (*unit*)	Inhibition (%)
0	—	57	—
3.0×10^4	1.5	59	0
6.0×10^4	—	51	10.5
1.2×10^5	11.7	40	29.8
2.6×10^5	15.4	28	50.8
4.8×10^5	31.8	11	80.7

1) 50 μg. of DPN was contained in 1 ml. of water.

2) The solution containing 50 μg. in 0.1 ml. of GDH of 12,000 units per mg. of protein was used to measure the activity of enzyme.

TABLE III

Effects of γ-Rays on Holoenzyme Systems of Glutamic Dehydrogenase

Holoenzyme system	Amount of enzyme (μg.)	Dose ($r\times10^{-4}$)	Inactivation of constituent[4] (%)	Inhibition of the activity of holoenzyme system (%)
GDH[1]	25	0.8	0	40
DPN[3]	(50)	15.0	40	
GDH[1]	25	2.5	32	84
DPN[3]	(50)	30.0	60	
GDH[2]	50	6.3	39	39
DPN[3]	(600)	6.3	17	

1) Apoenzyme solution containing 1.25 mg. per ml. of GDH of 200 units per mg. of protein was used.

2) Apoenyme solution containing 0.4 mg. per ml. of GDH of 1,200 units per mg. of protein was used.

3) Coenzyme solution containing 1.2 mg. of DPN per ml. in 0.2 M phosphate buffer was used.

4) Holoenzyme solution containing non-irradiated apoenzyme or coenzyme was used to determine the activity of its constituent of holoenzyme.

As may be seen from the table, the larger the ratio between apoenzyme and DPN, the stronger was the inhibitory effect of the irradiation, indicating that a substance(s) which existed in the DPN-preparation had a protecting action on the apoenzyme. Several substances were tested for their enzyme-protecting effects against the radiation products of water, and the results obtained are summarized in Table IV.

TABLE IV

Protecting Power of Several Compounds Against Inactivation of Glutamic Dehydrogenase

Compound	Concentration (M)	Amount added (*mg.*)	Protecting power Q[1]
Reduced glutathione	4.0×10^{-3}	0.6	1.29
DPN	$1\cdot6\times10^{-3}$	0.0833	$4\cdot37\times10^{-1}$
Phenylalanine	0·5	41.0	$1\cdot86\times10^{-2}$
KH_2PO_4	0.2	13.6	1.22×10^{-2}
Thiourea	0.5	19.0	5.68×10^{-3}
Sodium sulphate	0.5	35.0	$5\cdot53\times10^{-3}$
Ethanol	3.0	69.0	3.16×10^{-3}

1) Protecting power Q was defined by Dale (*13*) as;

$$Q=\frac{{}_{37}D_{p+e}-{}_{37}D_e}{{}_{37}D_e}\times\frac{W_e}{W_p}$$

Where ${}_{37}D_{p+e}$ is the dose required to cause 37 per cent inhibition of initial enzyme activity when the compound is added to the enzyme solution to be irradiated; ${}_{37}D_e$ is the same dose without compounds; W_e is the weight of enzyme, 90 μg. containing the activity of 1,200 units per mg. of protein in 0.1 ml. of the solution, and W_p is the amount of compound added to be irradiated.

TABLE V

Reactivation of γ-Irradiated Glutamic Dehydrogenase with Glutathione

Dose (r)	Activity inhibited before addition of glutathione[1] (*unit*)	Activity reactivated after addition of glutathione[2] (*unit*)	Inhibition (%)	Reactivation (%)
0	91	95	—	—
1800	85	92	6.6	100
12500	49	57	46.2	19.0

1) The enzyme solution contained 80 μg. in 0.1 ml. of GDH of 1,200 units per mg. of protein; 500 μg. of DPN in 0.5 ml. was added to measure the activity of enzyme.

2) Before measurement of the enzyme activity 2×10^{-3} M of reduced glutathione was added.

The protecting actions of denatured protein of the inactivated enzyme against inhibition of virginal glutamic dehydrogenase, were also observed. Extraordinarily strong protecting action of reduced glutathione against inactivation of liver glutamic dehyrogenase by γ-ray shown in Table V should be ascribed to the effect of its sulfhydryl group.

SUMMARY

1. The mechanism of inhibitory action of γ-ray upon various dehydrogenases was studied.

2. Alcohol dehydrogenase of yeast and glutamic dehydrogenase of beef liver were observed to be inhibited by relatively low doses of γ-ray.

3. Inactivation of liver glutamic dehydrogenase was independent of the amount of enzyme, provided that the enzyme concentration was not extraordinarily low.

4. The coenzyme of these dehydrogenases, DPN, was also found to be affected by γ-radiation, the changes in its molecular structure being demonstrated by the modification of its ultraviolet absorption; DPN was observed, however, to be more resistant than apoenzymes against γ-ray.

5. The effects of γ-rays on holoenzyme systems of glutamic dehydrogenase were examined, and it was found that the larger the ratio between apoenyme and DPN, the stronger was the inhibitory effect of the irradiation.

6. The protecting action of several compounds against inactivation of glutamic dehydrogenase by γ-irradiations was also studied. Reversible reactivation of inhibited glutamic dehydrogenase caused by the SH group of reduced glutathione was also observed.

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STUDIES ON PROTAMINES

VI. AMINO ACID COMPOSITION OF CLUPEINE AND SALMINE*

By TOSHIO ANDO, SHIN-ICHI ISHII and MITSUO SATO**

(*From the Department of Biophysics and Biochemistry, Faculty of Science, University of Tokyo, Tokyo*)

(Received for publication, December 25, 1958)

Numerous results of amino acid composition of salmine and clupeine have been reported (*2–11*), but it is rather difficult to find a good accord among these data for each of the protamines. The discrepancies have been attributed to differences in origin of the protamine samples, besides differences in analytical methods. Since heterogeneity of clupeine, salmine, and iridine has been demonstrated more distinctly by recent authors (*8*, *12–17*), it should also be considered, that the specimens prepared from the same origin by different procedures may show different compositions. Studies intended to prepare the homogeneous specimens of the protamines are now in progress in this laboratory, too. Some difficulties, however, still remain in the studies, because each of the protamines may be a mixture of closely related molecules. Most of the results stated in this series of reports are, therefore, on the heterogeneous specimens prepared by a definite procedure. The present paper shows the results obtained for the amino acid composition of those typical specimens, one of salmine and two of clupeine, by ion-exchange chromatography. Similar data of iridine have already been reported in this series (*18*).

EXPERIMENTAL

Material—Two specimens of clupeine were used: one was "the less soluble" clupeine sulfate prepared from mature milts of herring (*Clupea pallasii*) caught at a north-east coast of Hokkaido, Mombetsu, facing the Sea of Okhotsk on May 7, 1947 and was designated in this paper as clupeine I; the other was also "the less soluble" sulfate from herring of the same species caught at a west coast of Hokkaido, Yoichi, facing the Japan Sea on April 3, 1951 and was designated as clupeine II. Salmine specimen used was "the less soluble" sulfate prepared from salmon (*Oncorhynchus keta*) obtained in Hokkaido on October 2, 1950. Details on the preparation of those protamine sulfates have been described in a previous paper in this series (*15*).

Hydrolysis—Each hydrolysis of the protamine sulfates (18–23 mg.) was performed in a sealed glass tube in an oven at 110° for 32 hours with a 200-fold quantity of glass-redistilled HCl (6 *N*). In all cases, the hydrolysates were colorless and contained no insoluble humin. Excess HCl was removed by concentrating the hydrolysates repeatedly *in vacuo* at 40–50° and finally by leaving over NaOH pellets in a vacuum desiccator.

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* For the Paper V, see Ando *et al.* (*1*).

** Present address: National Institute of Industrial Health, Kawasaki, Japan.

Chromatographic Separation—The separation of neutral amino acids in the hydrolysates was carried out by chromatography on a column (0.9×100 cm.) of Dowex 50-X 8*, operated essentially as described by Moore and Stein (*19*). Each hydrolysate evaporated to dryness was dissolved in 0.1 *M* sodium citrate buffer of pH 3.42 (1.5–3 ml.) and the resulting solution was then adjusted to pH 2.5–3.0. An aliquot (0.5 ml.) containing about 1 mg. of nitrogen** from the solution was pipetted into the resin column, and elution was started with the buffer of pH 3.42. As the second eluent in the stepwise elution, pH 5.0 sodium citrate buffer (0.1 *M*) was sometimes used instead of the pH 4.25 buffer. The column was maintained at 38±0.5° throughout the elution.

The remainder of the hydrolysate solution was diluted 20-fold with the eluent buffer of pH 6.0, and an aliquot (0.5 ml.) containing about 0.05 mg. of nitrogen was added to a column of Amberlite IRC-50 (XE-64). By chromatography on this column, a basic amino acid, arginine, was separated from the other amino acids and ammonia. It was operated according to the description of Ishii (*20*), except that a shorter column of 0.6×10 cm. was used.

The effluent fractions from both columns were collected on an automatic fraction collector of the magnetic burette type (*21*).

Analysis of Effluent Fraction—The effluent fractins were adjusted to about pH 5 and analyzed, except for proline, by the ninhydrin photometry of Yemm and Cocking (*22*) on a Kotaki AKA photoelectric colorimeter with a 570 mμ filter. The concentrations of proline were estimated by the acidic ninhydrin method of Chinard (*23*) on a Beckman Model DU quartz spectrophotometer at 515 mμ.

Measurement of Acid Labilities of the Constituent Amino Acids—The following control experiments were performed to estimate the degrees of decomposition of the constituent amino acids under the hydrolytic condition. Samples of DL-serine, L-threonine, L-proline, L-arginine hydrochloride, glycine, DL-alanine, DL-valine, and L-isoleucine were heated separately in sealed tubes with a 200-fold quantity of 6 *N* HCl for 32 hours at 110°. The excess HCl was removed by repeated concentration *in vacuo* and the residues were dissolved in water. The NH_3 content of each resulting solution was determined by the microdiffusion method of Cónway (*24*).

Discrimination between Isoleucine and Leucine—One-dimensional paper chromatography with water-saturated *tert*-amyl alcohol (*25*) was employed to decide whether isoleucine or leucine is present as a component of salmine and clupeine. Salmine and clupeine II (10 mg. of each sulfate) were hydrolysed, for this experiment, in a similar manner as described above. After excess HCl was removed, the hydrolysates were dissolved in water. An aliquot containing about 0.12 mg. of nitrogen from each resulting solution was applied on Whatman No. 1 paper (46 cm. in length), chromatographed by a descending technique at room temperature (17–19°), and revealed by ninhydrin reaction. In the developing solvent, water-saturated *tert*-amyl alcohol, 0.04 per cent 8-hydroxyquinoline was added in order to eliminate a disturbing effect by traces of copper ions in the paper.

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* The resin used was a fraction which passed through a 150 mesh sieve and did not pass through a 250 mesh sieve in the wet sodium form.

** The amount of the hydrolysate added to the column was determined by micro-Kjeldahl analysis on another aliquot of the solution. The nitrogen contents of the present specimens of clupeine sulfates I and II and salmine sulfate are 24.0, 23.7_5, and 23.3 per cent, respectively (*15*).

RESULTS AND DISCUSSION

Figs. 1 and 2, respectively, show a typical elution diagram of a hydrolysate of salmine from a Dowex 50 column and that of a hydrolysate of clupeine I

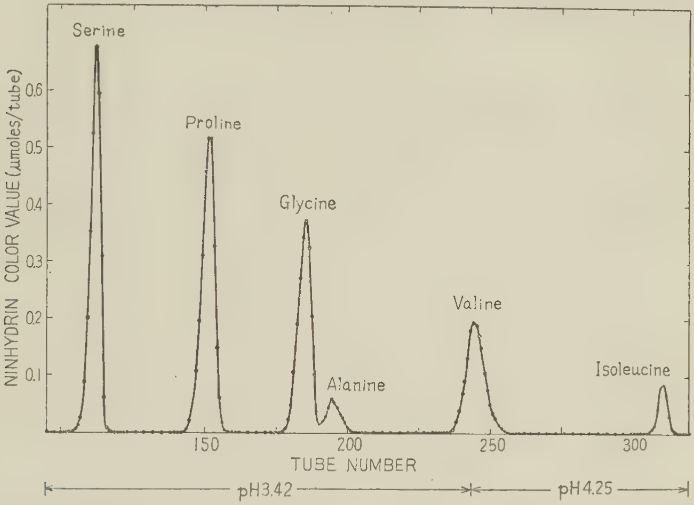

FIG. 1. Chromatographic separation of neutral amino acids in a hydrolysate of salmine (1.44 mg. as the amount of total N) on a 0.9×100 cm. column of Dowex 50-X 8. The column was maintained at 38±0.5° throughout the elution, and the effluent was collected in 0.99 ml. fractions. The ninhydrin color values, for all the amino acids except proline, are shown as isoleucine equivalents.

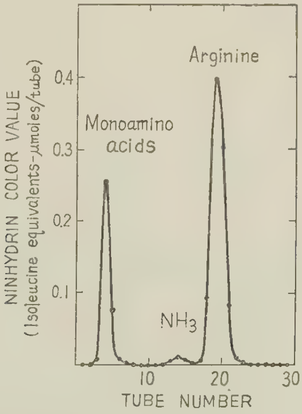

FIG. 2. Chromatographic separation of arginine in a hydrolysate of clupeine I (45.2 μg. as the amount of total N) on a 0.6×10 cm. column of Amberlite IRC-50 (XE-64). The column was maintained at 30±0.5° throughout the elution, and the effluent was collected in 0.88 ml. fractions.

from an Amberlite IRC-50 (XE-64) column. As may be seen in Fig. 2, such a small column of 0.6×10 cm. is sufficient to separate arginine from ammonia and monoamino acids. The analytical data for the protamine hydrolysates are shown in Table I. Before comparing these values with each other, attention should be paid to the adequacy of the hydrolytic condition used in the present experiments and the lability of some amino acids during hydrolysis.

TABLE I

Amino Acid Composition of Hydrolysates of Clupeine and Salmine

Amino acid	Amino acid N as a percentage of total N		
	Clupeine I[1]	Clupeine II[2]	Salmine[1]
Glycine	0.57	0.22	2.1
Alanine	2.7	2.5	0.32
Valine	1.2	1.7	1.6
Isoleucine	0.57	0.27	0.34
Serine	2.2	2.3	2.9
Threonine	1.0	0.94	0.0
Proline	2.5	2.5	2.9
Arginine	89.0	88.0	89.7
Ammonia[3]	—	0.46	0.49
Total		98.9	100.3

1) Average values from two determinations.

2) Values from a single determination.

3) Estimated on unfractionated hydrolysates by the microdiffusion method of Conway (*24*).

The hydrolytic condition of heating for 32 hours was adopted after following the rate of liberation of amino nitrogen from clupeine, under heating at 110° with 6 *N* hydrochloric acid, by the manometric method of Van Slyke (*26*)*. But it has been reported by several authors (*27–29*) that heating time longer than 32 hours is frequently necessary to insure complete liberation of isoleucine and valine from their peptide linkages that may be especially resistant to hydrolysis. Accordingly, there may be a slight increase in the values of these amino acids in Table I.

From the results of experiments carried out to determine the acid lability of all the constituent amino acids, it was observed that only serine, threonine, proline, and arginine decomposed in a detectable degree under the hydrolytic condition. These amino acids showed 11, 3.5, 0.6, and 0.08 per cent losses respectively, on the basis of one mole ammonia formed from one mole of the

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* The measurement of free amino nitrogen was kindly made by Dr. M. Kimura in this laboratory (present laboratory: National Institute of Industrial Health).

TABLE II

Amino Acid Composition of Clupeine and Salmine

Amino acid	Amino acid N as a percentage of total N					
	Clupeine			Salmine		
	Present work		Waldschmidt-Leitz *et al.* (*11*)[3]	Present work	Corfield *et al.* (*7*)[4]	Callanan *et al.* (*8*)[5]
	Clup. I[1]	Clup. II[1]				
Glycine	0.57	0.22	0.3	2.1	1.80	1.96
Alanine	2.7	2.5	2.4	0.32	0.45	0.55_8
Valine	1.2	1.7	1.4	1.6	1.40	1.42
Isoleucine	0.57	0.27	0.3	0.34	0.44	0.38_3[6]
Serine	2.5[2]	2.6[2]	2.6	3.3[2]	3.12	3.04
Threonine	1.0[2]	0.97[2]	1.0			
Proline	2.5	2.5	1.9	2.9	2.70	3.13
Arginine	89.0	88.0	89.0	89.7	89.8	87.7
Total	100.1	98.8	98.9	100.2	99.71	98.19
Arg/ Others[7]	2.02	2.05		2.12		
Minimal mol. wt.[8]	7,800	20,000		13,800	10,000	12,000

1) See experimental part.

2) Corrected for decomposition during hydrolysis by the factors of 100/89 for serine and 100/96.5 for threonine.

3) Average (computed by the present authors) of data of three specimens (III, IV and V in Table II of reference (*11*)) from European herring (*Clupea harengus*).

4) Data of a commercial specimen (Boots Pure Drug Co., Ltd.).

5) Data of a specimen from a species of Pacific salmon (*Oncorhynchus tschawytscha*).

6) A value for leucine.

7) Molar ratio of arginine to all the monoamino acids.

8) Minimal molecular weight (as a free base), temporarily calculated on the assumption that one residue of the least component amino acids would be contained in each molecule.

amino acids decomposed*. Hence the correction factors for the losses were computed to be 100/89 for serine, 100/96.5 for threonine, 100/99.4 for proline, and

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* Rees (*30*) has found that serine and threonine, when refluxed with 6N hydrochloric acid, decompose to the extents equivalent to ammonia liberated from each amino acid. In the present experiments, the remaining amounts of the amino acids after the hydrolytic treatment were not determined directly, but it was verified by paper chromatography that all the amino acids tested, never changed to other amino acids (or ninhydrin-positive substances) during the treatment. Therefore, although there can be seen some objections (*31*, *32*) against above findings of Rees in the case of threonine, the present authors accepted the assumption, that the lost amounts of the amino acids may correspond to the measured amounts of ammonia.

$100/99.9_2$ for arginine. Compared with the well-known data of Rees (*30*), a somewhat small correction factor is obtained for threonine in the present experiment, while a good accord can be seen with serine. Values of serine and threonine corrected by these factors were shown in Table II, where the results of the present analyses were summarized. The correction factors for proline and arginine were neglected, since analyses were not sufficiently accurate to warrant their use.

At the bottom line in Table II is given the minimal molecular weight of each protamine, temporarily calculated on the assumption, that one residue of the least component amino acid would be contained in each molecule. All of these molecular weights are larger than the values found previously in this laboratory on the same specimens by end group analyses*. The discrepancy may be elucidated best by considering heterogeneity of the specimens of protamines.

It has been reported by this laboratory, (*34*, *35*) that each specimen of clupeine is a mixture of molecules having an alanine residue and those having a proline residue, on their N-termini, and that the mixed ratio differs among such specimens as the place or date of capture of fish material differs. Clupeine I used in this study has a large ratio (alanine/proline on an N-terminus) compared with clupeine II. It was expected at first, therefore, that some apparent difference between amino acid compositions of both clupeines would be found in the values of alanine and/or proline. But it was not the case. As shown in Table II, a significant difference exists only in the contents of glycine, valine, and isoleucine. Each amount of isoleucine and glycine, both of which are the least component in each specimen, is larger in clupeine I than in clupeine II. On the other hand, the amount of valine is less in clupeine I.

Though clupeine I and clupeine II were prepared by a principally same method, there must have been a certain degree of difference between them in details of the actual operations. It may be taken into consideration, that this difference can be at least one of the causes of the differences between their compositions.

The presence of glycine was reported, several years ago, by this laboratory (*36*) in specimens of clupeine from Japanese herring (*Clupea pallasii*). Up to that time, few researchers (*37*, *38*) had recognized this amino acid as a component of clupeine from the European herring (*Clupea harengus*). Not only about the presence of glycine but also about their N-termini (*39*), there had been distinct differences between the results obtained with clupeine specimens from both species. But now, these discrepancies seem to be diminished. European researchers (*11*, *14*, *16*) have recently noticed the presence of glycine. The amino acid composition of our clupeine II is quite similar to that of European clupeine recently reported by Waldschmidt-Leitz *et al.* (*11*).

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* Molecular weight of both clupeine and salmine (as a free base) has been estimated to be about 6,000 by the carboxyl end group determination (*33*) and to be about 4,800 by the amino end group determination (*34*).

As may be seen in Table II, the differences between these two results, especially in amounts of the least two components, are rather less than those between the results of our two clupeines, I and II, which were prepared from materials of the same species.

As to amino acid composition of salmine, numerous results have already been reported for various specimens. While most of the researchers have stated that salmine has isoleucine as one of the components, Tristram (*2*) and Callanan *et al.* (*8*) have reported that leucine rather than isoleucine occurs. The determination, therefore, of the presence of leucine or isoleucine in our salmine should be necessary. The elution chromatogram in Fig. 1 shows a single peak which seems to be due to the presence of either leucine or isoleucine, at the effluent volume of about 310 ml.; but that which of the two it is due to, can hardly be decided only by its position. The final solution for the present problem was obtained by one-dimensional paper chromatography with *tert*-amyl alcohol as a developing solvent (*25*): our salmine contains isoleucine ($R_f=0.29$), and not leucine ($R_f=0.35$). As to clupeine, although there was no doubt left on that problem by our previous investigation (*15*), further inquiry was tried by the same method. In this case also, isoleucine was confirmed to be the component.

From the quantitative view-point, the present result on the amino acid composition of salmine shows the closest resemblance to those obtained by Corfield and Robson (*7*) and by Callanan *et al.* (*8*), although there are some differences in species among specimens used. The comparison among these three data is given in Table II.

SUMMARY

Quantitative amino acid analyses of specimens of clupeine (*Clupea pallasii*) and salmine (*Oncorhynchus keta*) have been made by ion-exchange chromatography. Glycine, alanine, valine, isoleucine, serine, threonine, proline, and arginine for clupeine, and glycine, alanine, valine, isoleucine, serine, proline, and arginine for salmine were identified and determined.

Some differences in the contents of glycine, isoleucine, and valine could be found between two specimens of clupeine, which differ in the place or date of capture of their fish material.

The present results of both protamines were compared with the previous data of those from different species reported by other authors.

The authors are indebted to Dr. M. Minagawa for his cooperation in the perliminary analysis by ion-exchange chromatography, and to Dr. K. Iwai for his valuable discussion. The present work was aided in part by the Scientific Research Grant from the Ministry of Education.

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THE METABOLISM OF *p*-METHYLPHENYLALANINE AND *p*-METHOXYPHENYLALANINE

II. THE METABOLISM OF *p*-METHYLPHENYLPYRUVATE

By KYOSUKE UMEZAWA, YUKIYA SAKAMOTO AND KATASHI ICHIHARA

(*From the Biochemical Department, Medical School, Osaka University, Osaka*)

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Dakin *et al.* (*1*) reported that *p*-methylphenylalanine and *p*-methoxyphenylalanine were converted to acetoacetic acid in organisms without the formatin of homogentisic acid. In these experiments, the reduction of iodine was determined in the distillate of the reaction mixture from a perfused liver. Reduction value was presumed to indicate the amount of acetoacetic acid. According to previous papers of Ichihara *et al.* (*2*) the distillate from a liver extract of a rat after incubation with *p*-methylphenylalanine or *p*-methoxyphenylalanine reduced iodine but acetoacetic acid could not be demonstrated by the aniline-citrate method. Moreover *p*-methylphenaceturic acid was isolated from the urine of a cat after administration of *p*-methylphenylalanine. Recently Crandall *et al.* (*3*) reported an increase of acetoacetic acid during perfusion of liver with *p*-methoxyphenylalanine, and attributed the increase of acetoacetic acid to the ketogenic action of ammonia which was produced from this amino acid. According to report of Annau (*4*) ammonia has an effect to increase acetoacetic acid formation.

As described in the previous papers, Icihara *et al.* used phenylpyruvate. The authors carried out the experiment using *p*-methylphenylpyruvate as described below.

EXPERIMENTALS

Material—*p*-Methylphenylpyruvic acid (m.p. 174°) was prepared by hydrolyzing the azlactone synthesized according to the method of Herbst (*5*). DL-*p*-methylphenylalanine (m.p. 277–279°) was synthesized by the method of Okuda and Fujii (*6*) and *p*-methylphenaceturic acid was isolated by the method described by Ichihara *et al.* in previous papers.

Method 1—2.0 g. *p*-methylphenylpyruvic acid were dissolved in 100 ml. of distilled water containing 20 ml. of a saturated sodium carbonate solution. The solution was adjusted to pH 7.2 with 10 per cent hydrochloric acid. 10 ml. of this solution were injected subcutaneously at three hour intervals into a male rabbit (body weight 2.5 kg.). All the urine from the beginning of the injections until 48 hours after the last injection was collected in a bottle containing 20 ml. of 10 per cent sulfuric acid. One tenth of the urine was used for paper chromatography and the rest for the isolation of *p*-methylphenaceturic acid.

Method 2—One tenth of the urine was neutralized with 10 per cent sodium hydroxide and evaporated at 40° in vacuo to a syrup. Alcohol was added to the syrup, and the alcohol extract was evaporated at 40° and used for paper chromatography. As developing solvent glacial acetic acid : butanol : water (1 : 4 : 1) was used, and as paper Toyo filter paper No. 51. After development, ninhydrin or ferric chloride solution was sprayed on the paper to develop the spots.

RESULTS

I) With ninhydrin a spot of R_f 0.55 was detected which was in the same position as that of an authentic sample of *p*-methylphenylalanine. By ferric chloride a spot at R_f 0.71 was found, which accorded in position with that of *p*-methylphenylpyruvate. After the sample had been acidified with 10 per cent hydrochloric acid and heated at 100° for 60 minutes the hydrolyzate was neutralized with 30 per cent sodium hydroxide and concentrated at 40° under reduced pressure and used for paper chromatography. By ninhydrin, a spot at R_f 0.19, according with that of glycine was detected.

II) The urine of a rabbit which had been given 2 g. of *p*-methylphenylpyruvate subcutaneously, was acidified with 10 per cent sulfuric acid and extracted twice with two volumes of ether. When the ether extract was evaporated, a crystalline mass (*ca.* 800 mg.) was obtained. This was almost insoluble in water, and on paper chromatography a spot at R_f 0.71 was detected with ferric chloride.

After the crystals had been removed, the filtrate was acidified with 10 per cent sulfuric acid and extracted twice with two volumes of chloroform. The chloroform extract was evaporated to a syrup, and to this syrup acetone was added. When ligroine was added to the acetone extract a brown resin-like substance appeared on the wall of the beaker. The clear acetone-ligroine mixture was decanted into another beaker, and kept in a refrigerator for 24 hours. A crystalline substance (*ca.* 40 mg.) was obtained. It was recrystallized from acetone and ligroine and 15 mg. of pure crystals (m.p. 150 157°) were obtained. There was no depression of the melting point when these crystals were mixed with *p*-methylphenaceturic acid. 5 mg. of the crystals were dissolved in 5 ml. of 10 per cent hydrochloric acid and heated at 100° for 60 minutes and then used for paper chromatography. With ninhydrin a spot of glycine (R_f 0.19) was detected. Thus the crystals were identified as *p*-methylphenaceturic acid.

DISCUSSION

Ichihara *et al.* (*2*) reported previously that *p*-methylphenylalanine and *p*-methoxyphenylalanine were decomposed in an organism after the general rule of abbreviation of the side chain without opening of the benzene nucleus. This was in opposition to Dakin's opinion (*1*). In our previous study the compound which Dakin regarded as acetoacetic acid was suspected to be *p*-methylphenylpyruvate or *p*-methoxyphenylpyruvate. In these experiments on the basis of ability to be steam-distilled and to reduce iodine phenylpyruvate was used in order to duplicate the chemical behavior of *p*-methylphenylpyruvate and *p*-methoxyphenylpyruvate, since these two compounds were not available. In this paper, *p*-methylphenylpyruvate which was synthesized recently was used to confirm our previous assumption, and the fate of this compound in the rabbit was studied in detail. From previous and the above data the authors can conclude that *p*-methylphenylalanine, and perhaps *p*-methoxyphenylalanine, is converted by transamination to its corresponding pyruvate compound, and after the side chain of the latter is shortened to acetic acid and conjugated with glycine, excreted in the urine as *p*-methylphenaceturic acid. Most of the *p*-methylphenylpyruvate administered subcutaneously to a rabbit was excreted unchanged in the urine, but some was converted to *p*-methylphenylalanine by transamination, or to *p*-methylphenylacetic acid by decarboxylation.

SUMMARY

1. *p*-Methylphenylpyruvate administered to rabbits is excreted in the urine mostly unchanged. Some part is excreted as *p*-methylphenylalanine and *p*-methylphenaceturic acid. The former was demonstrated by paper chromatography and the latter by isolation of the crystalline compound.

2. *p*-Methylphenylalanine acts as a phenylalanine antagonist in the nutrition of rat. This antagonism is prevented by the addition of phenylalanine as described in the addendum.

Addendum

According to Wooley *et al.* (*7*, *8*) thienylalanine acts as a phenylalanine antagonist and 5-methyltryptophan as a tryptophan antagonist in the nutrition of animals. While studying the metabolism of *p*-methylphenylalanine the authors also carried out nutritional experiments with it.

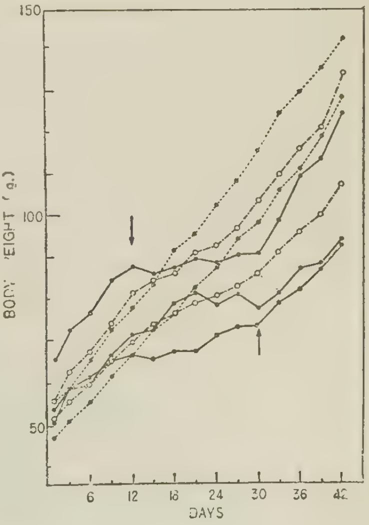

FIG. 1. Nutritional antagonistic effect of *p*-methylphenylalanine on phenylalanine.

All rats used in this experiment were fed on basal diet for first 12 days.

—·—○—·—: Following 18 days on diet containing 1.0 g. *p*-methylphenylalanine plus 1.0 g. L-phenylalanine/kg. body weight/day and on basal diet thereafter.

——●——: Following 18 days on diet containing 1.0 g. *p*-methylphenylalanine/kg. body weight/day and on basal diet thereafter.

—·—●—·—: Basal diet.

Young albino rats were fed on a basal diet for 12 days, and well grown rats of them were used. The basal diet had the following composition: 11.4 g. starch, 20 g. sugar, 2.0 g. casein, 0.4 g. McCollum salt, 0.1 g. oryzanin powder, 0.4 g. agar-agar, 2.0 g. butter and 20 ml. water. To the basal diet 1.0 g. of DL-*p*-methylphenylalanine or 1.0 g. DL-*p*-methylphenylalanine plus 1.0 g. L-phenylalanine was added per kg. body weight per day. As Fig. 1 shows, the growth of rats fed on the basal diet is inhibited by the addition of *p*-methylphenylalanine, and this inhibition is prevented by phenylalanine.

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The Journal of Biochemistry, Vol. 46, No. 7, 1959

SYNTHESIS OF α-*N*-BENZOYL-L-CANAVANINAMIDE AND ITS HYDROLYSIS BY TRYPSIN AND PAPAIN

By SEIICHIRO NAKATSU

(*From the Department of Chemistry, Faculty of Science, Kyushu University, Fukuoka*)

(Received for publication, February 2, 1959)

α-*N*-Benzoyl-L-argininamide (BAA) is known to be a typical synthetic substrate for trypsin and papain. It is interesting, therefore, to study the behaviors of these two proteolytic enzymes on α-*N*-benzoyl-L-canavaninamide (BCA) which has a similar molecular structure to that of α-*N*-benzoyl-L-argininamide. In the present work, the synthesis of α-*N*-benzoyl-L-canavaninamide was carried out, and its hydrolysis by trypsin and papain was investigated comparing with that of α-*N*-benzoyl-L-argininamide.

MATERIALS AND METHODS

Materials—L-Canavanine sulfate was prepared from jack bean according to Fearon and Bells's method (*1*). α-*N*-Benzoyl-L-argininamide was synthesized by the usual method. The trypsin and papain used were crystallin preparations of Nutritional Biochemicals Corporation (U.S.A) and Worthington Biochemical Corporation (U.S.A) respectively. The buffers employed were 1/5 *M* acetate, 1/15 *M* phosphate, and 1/20 *M* tris (hydroxymethyl) aminomethane (Tris).

Measurement of Enzyme Activity—Reaction mixtures (total volume 2 ml.) contained enzyme, substrate, buffer, and furthermore, in the case of papain, cysteine (0.005 *M*) and ethylenediamine tetraacetic acid (EDTA, 0.001 *M*). The reactions were run at 30°, and the amount of liberated ammonia was determined by Conway's method at appropriate intervals. The rates of hydrolysis of the substrates by the enzymes were first order reactions under the conditions of assay, and the enzyme activities were expressed as proteolytic coefficient (*C*, first order rate constant calculated in decimal logarithms per mg. of protein nitrogen per ml. of reaction mixture). The Michaelis constant (K_m) and the rate constant (k_3) for the conversion of enzyme-substrate complex to enzyme and products were calculated from Lineweaver and Burk's equation by using the method of least squares.

RESULTS AND DISCUSSION

*Synthesis of α-N-Benzoyl-*L*-canavaninamide*

*α-Benzoyl-*L*-canavanine*—To a chilled solution of 2.92 g. (10 mM) of L-canavanine sulfate and 1.06 g. (10 mM) of sodium carbonate in 17 ml. of water were added with vigorous stirring 1.55 g. (11 mM) of benzoyl chloride in several portions and a solution of 1.27 g. (12 mM) of sodium carbonate in 12 ml. of water. The addition of the reagents required about 1 hour, and the stirring was continued about for 2 hours thereafter. The reaction mixture was then acidified to about pH 2.5 with hydrochloric acid, and a slight precipitate formed here was filtered off. The filtrate was extracted three times with 30 ml. of ether, and the ether layers were discarded, and the aqueous layer was neutralized to about pH 6.0 with sodium hydroxide. Upon concentrating under diminished pressure, crystallization occurred. After being concentrated to about 10 ml. and kept in a refrigerator overnight, the crystals were collected. The yield was 1.6 g. (57 per cent). After recrystallization from hot water, the melting point (decomposition) was 215°, and $[\alpha]_D^{15}$ was $-31°$ (c 2.0, 5 *N* HCl).

Analysis Calcd. for $C_{12}H_{16}O_4N_4$: C, 51.42; H, 5.75; N, 19.99 per cent.
Found: C, 51.39; H, 5.77; N, 20.12 per cent.

α-N-Benzoyl-L-canavaninamide Hydrochloride—A suspension of 1.4 g. (5 mM) of the above compound in 65 ml. of absolute ethanol was chilled in an ice-bath, and was saturated with dry hydrogen chloride. After being kept at room temperature overnight, the solution was concentrated to a syrup under diminished pressure, and the residue was reesterified in the same manner. The resulting syrup was dissolved in a small volume of ethanol, and was precipitated by the addition of ether. The syrupy material obtained, probably α-*N*-benzoyl-L-canavanine ethyl ester hydrochloride, was used in the next step without further purification.

The above syrup was dissolved in 25 ml. of methanol that was previously saturated with dry ammonia at 0°. After being kept at room temperature for 2 days, the solution was evaporated under diminished pressure. The syrupy residue was dissolved in a small volume of water, and was treated with silver hydroxide (5 mM). The silver chloride formed was filtered off, and the filtrate was evaporated under diminished pressure. (By this procedure, the ammonium chloride which was contained in the syrup could be removed completely). The resulting syrup was dissolved in 5 ml. of water, and the solution was again evaporated under diminished pressure after acidification to about pH 2.5 with hydrochloric acid. The residue was dissolved in 2.5 ml. of ethanol, and was precipitated by the addition of 7.5 ml. of ether. After several days in a refrigerator, the material crystallized. After recrystallization from 2.5 ml. of hot ethanol, the yield was 0.6 g. (38 per cent), the melting point 170°, and $[\alpha]_D^{15} -15°$ (c 2.0, H_2O).

Analysis Calcd. for $C_{12}H_{17}O_3N_5 \cdot HCl$: C, 45.65; H, 5.74; N, 22.18 per cent.
Found: C, 45.50; H, 5.68; N, 21.87 per cent.

Hydrolysis by Trypsin

It was found that α-*N*-benzoyl-L-canavaninamide was hydrolysed very slowly by trypsin as compared with α-*N*-benzoyl-L-argininamide at pH 7.8 (the optimal pH of the enzyme), as shown in Table I. Since canavanine has a molecular structure similar to that of arginine, this result seems curious, but may show the fact that trypsin has a very sharp specificity.

TABLE I

C for Hydrolysis of BAA and BCA by Trypsin

Substrate	C
BAA	0.20
BCA	0.001

Trypsin 0.05 mg. protein N/ml., substrate 0.01 *M*, 1/20 *M* Tris buffer (pH 7.8), 30°.

Hydrolysis by Papain

Because, in contrast with the case of trypsin, the rate of hydrolysis of α-*N*-benzoyl-L-canavaninamide by papain was found to be in the same order as that of α-*N*-benzoyl-L-argininamide, the reaction was studied in detail.

Effect of pH—The effect of pH on the rate of hydrolysis of α-*N*-benzoyl-L-canavaninamide by papain is shown in Fig. 1. The shape of pH-activity curve is similar to that for α-*N*-benzoyl-L-argininamide reported by Kimmel and Smith (*2*), and the enzyme has the maximal activity near pH 6.

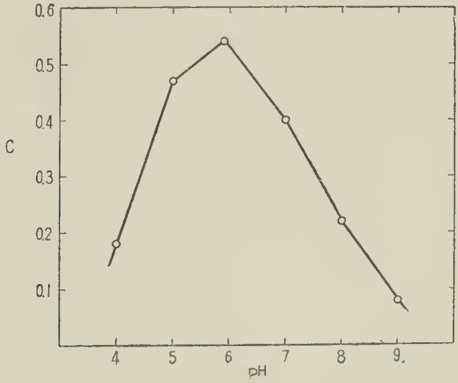

FIG. 1. pH-activity curve for hydrolysis of BCA by papain. papain 0.01 mg. protein N/ml., BCA 0.01 *M*, cysteine 0.005 *M*, EDTA 0.001 *M*, 30°.

Kinetic Constants—The values of C at various initial substrate concentrations for the hydrolysis by papain are given in Table II with the values of K_m and k_3 calculated from these data. The fact that the values of K_m and k_3 for α-*N*-benzoyl-L-canavaninamide are smaller than those for α-*N*-benzoyl-L-argininamide may suggest that the former is bound more tightly, but is hydrolyzed more slowly by papain than the latter. (The values of k_3 obtained here may be minimal values, because there is the possibility that the papain used contained some inert proteins).

TABLE II

Kinetic Constants for Hydrolysis of BAA and BCA by Papain

Substrate	C				K_m[1]	k_3[2]
	0.005 M	0.01 M	0.02 M	0.05 M		
BAA	0.88	0.80	0.67	0.52	0.066	0.14
BCA	0.60	0.50	0.42	0.29	0.041	0.060

Papain 0.01 mg. protein N/ml., 1/15 M phosphate buffer (pH 5.9), cysteine 0.005 M, EDTA 0.001 M, 30°.

1) mole.

2) mole/litre/min./mg. protein N/ml.

SUMMARY

1. α-N-Benzoyl-L-canavaninamide hydrochloride was synthesized from L-canavanine sulfate through α-N-benzoyl-L-canavanine and its ethyl ester hydrochloride.

2. The rate of hydrolysis of this compound by trypsin was about 1/200 of that of α-N-benzoyl-L-argininamide at pH 7.8 and 30°.

3. The rate of hydrolysis of the compound by papain was about 2/3 of that of α-N-benzoyl-L-argininamide at pH 5.9 (the optimal pH) and 30°, and the Michaelis constant for the reaction was 0.041 M.

The author wishes to thank Prof. Dr. S. Shibuya for his kind encouragement and advice, and Assistant Prof. Dr. N. Izumiya for his generous gift of trypsin, papain, and many other materials and valuable advice, and Miss H. Okazaki for her synthesis of BAA.

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ENZYMIC OXIDATION OF CARBON MONOXIDE
II

By TATSUHIKO YAGI

(*From the School of Medicine, Tokyo Medical and Dental University, Tokyo*)

(Received for publication, February 2, 1959)

Since the beginning of this century, there are a number of reports on the utilization of carbon monoxide by microorganisms (*1–12*). Some of them deal with its oxidation (*4*, *5*, *9*, *11*), while others with its reduction (*6–8*, *10*). There are autotrophs which can live on carbon monoxide as the sole source of carbon (*3*, *5*, *11*). Recently Kistner reported the adaptive nature of the cellular activity of oxidizing carbon monoxide (*12*).

In the previous paper (*13*), it was reported that carbon monoxide was oxidized into carbon dioxide in the presence of cell free extracts of *Desulfovibrio desulfuricans*. Sulfite was used as an oxidant.

The activity can be ascribed to the presence of an enzyme, the nature of which is to render active or unstable carbon monoxide to be oxidized in the presence of a suitable electron acceptor. The present paper deals with some properties of this enzyme.

MATERIALS

Bacteria—Desulfovibrio desulfuricans, strain I, was used as an enzyme source. This is the same strain which Ishimoto *et al.* (*14*) used in their work on sulfate reduction. The organism was grown anaerobically in five-liter flasks in a medium containing: 0.8 per cent lactic acid, 0.3 per cent Na_2SO_4, 0.03 per cent $MgSO_4 \cdot 7H_2O$, 0.015 per cent KH_2PO_4, 0.2 per cent polypeptone *Takeda*, 0.1 per cent meat extracts *Mikuni*, and tap water to the volume, pH being near 7. A few drops of saturated ammonium polysulfide solution was added in some of the experiments.

The cells grown after an incubation of 48 to 72 hours at 37°, were harvested by centrifugation, washed three times with distilled water, and suspended in distilled water at a concentration of 2 to 2.5 g. wet cells per 10 ml. The suspension was stored in frozen state.

Bacterial Extracts—Bacterial extracts were prepared by subjecting the cell suspension to sonic disintegration for ten minutes followed by centrifugation at 17,000 × *g* for an hour. The extracts thus obtained contained 15 to 30 mg. organic matter (*15*) per ml. In some cases extracts were prepared by grinding cells with alumina.

Carbon Monoxide—Carbon monoxide was prepared by decomposing formic acid by concentrated sulfuric acid.

EXPERIMENTALS AND RESULTS

Method for the Assay of Carbon Monoxide Activating Enzyme—The activity can be demonstrated and assayed in a system in which uptake of carbon monoxide is measured manometrically in the presence of benzyl-viologen as an electron acceptor and of alkaline absorbent in the center well. The system is by far simpler than the one reported earlier (*13*) in which sulfite was used as an electron acceptor, because we can avoid the effect of sulfite reductase (*16*). The improvement enabled us to undertake experiments giving more detailed information on the nature of the enzyme.

In the main compartment of a Warburg vessel were placed properly diluted bacterial extracts and 75 μ moles phosphate buffer pH 7. To the side arm, 20 μ moles benzyl-viologen and a few mg. zinc dust were added. The total volume of the reaction medium was adjusted to 1.5 ml. Gas phase in the manometer was 100 per cent carbon monoxide. The temperature was 30°. After equilibration, benzyl-viologen, which was partially reduced by the zinc dust, was tipped in to the main compartment leaving heavy lumps of zinc metal in the side arm. In case the blue color of the partially reduced dye disappeared, the reaction mixture was again subjected to contact with zinc metal in the side arm so as to maintain the reaction mixture faintly blue. As shown in Fig. 1 the rate of gas uptake is proportional to the amount of the enzyme added. The activity was expressed in Q_{co} (μl./hr./mg.). In this method the effect of zinc is minimizing an irregular delay. The extracts used in these experiments were found to contain active formate and pyruvate dehydrogenases. The addition of sodium formate or pyruvate instead of zinc was, therefore, also effective to minimize the time of lag. In some experiments, in the absence of such reducing agents, no activity was observed. Typical two examples are illustrated in Fig. 2. The boiled extracts and the dialyzate did not catalyze the reaction.

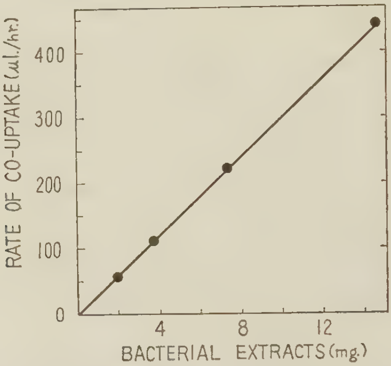

FIG. 1. Relation between the rate of gas uptake and the amount of the bacterial extracts added. Conditions are described in the text.

The Effect of Heat Treatment—The rate of heat inactivation of the enzyme was compared with those of hydrogenase and formate dehydrogenase at various temperatures. The results are given in Table I.

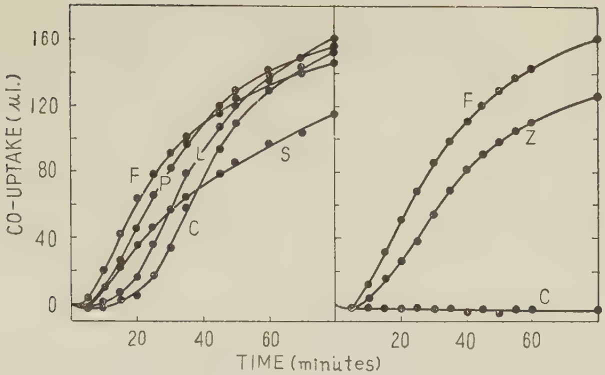

FIG. 2. Effect of reducing agents to initiate the gas uptake. Each vessel contained 0.5 ml. extracts (16 mg./ml.), 75 μM phosphate buffer, pH 7 and 20 μM benzyl-viologen. 1 μM sodium formate was added to F. 1 μM sodium pyruvate to P, 1 μM lithium lactate to L, 0.2 mg. sodium hydrosulfite to S and a few mg. zinc dust to Z. Curve C is the control with no addition. Total volume of the mixture was 1.5 ml. The gas phase was 100 per cent carbon monoxide. Temperature was 30°.

TABLE I

The Effect of Heat Treatment

		Before the treatment	70° 20 min.	80° 20 min.	100° 30 min.
CO activating enzyme	(*μl./hr./ml.*)	50	27	16	0
	(%)	(100)	(54)	(22)	(0)
Hydrogenase	(*μl./hr./ml.*)	2000	290	26	0
	(%)	(100)	(14)	(1.3)	(0)
Formate dehydrogenase	(*μl./hr./ml.*)	270	10	<0.7	0
	(%)	(100)	(4)	(<0.2)	(0)

Bacterial extracts were heated as designated in this table and centrifuged. The supernatant was used for the assay. Hydrogenase was assayed manometrically by the method of Tamiya *et al.* (*17*). Formate dehydrogenase was assayed by the decolorization of methylene blue in the presence of formate in a Thunberg tube.

The values in parentheses give the remaining activity in per cent when the activity values before the treatment were taken as 100 per cent.

The Effect of Hydrogen Ion Concentration—The relation between pH and the rate of gas uptake was tested. The results are given in Fig. 3. Optimum pH range was 7 to 8.

The Effect of Light and of the Partial Pressure of Carbon Monoxide—The effect of illumination on the reaction rate at various partial pressure values of carbon monoxide in the gas phase was examined. The condition and the results are shown in Table II.

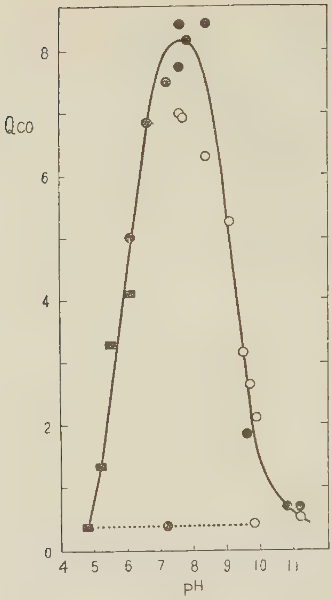

FIG. 3. Effect of pH on the reaction rate.

Conditions were similar to those described in the text except for that phthalate buffer (■), phosphate buffer (●) or glycine-NaOH buffer (○) was employed. Heated extracts (100° 40 minutes) had no activity as shown by the broken line in the figure.

TABLE II

The Effect of Light and of the Partial Pressure of Carbon Monoxide

Partial pressure of CO (*atm.*)	Exp. No.	Dark	Light	Light/Dark
1	Exp. 1	100	85	
	Exp. 2		95	
	Exp. 3		92	
	(Average)		(91)	(0.91)
0.1	Exp. 1	95	67	
	Exp. 2	102	88	
	Exp. 3	77	78	
	(Average)	(91)	(78)	(0.85)
0.01	Exp. 1	32	28	
	Exp. 2	45	45	
	Exp. 3	44	23	
	(Average)	(40)	(32)	(0.80)

Partial pressure of carbon monoxide in the gas phase was adjusted by the addition of nitrogen.

In Experiment 2, each vessel contained 1.5 ml. reaction medium as described in the text. In Experiments 1 and 3, twice as much reaction mixture was used. The light vessels were illuminated by a 300 w. reflector lamp from a distance of 30 cm. in Experiment 1, and 25 cm. in Experiment 2. In Experiment 3, the vessels were illuminated by two 300 w. lamps from a distance of 20 cm. The rate of gas uptake in the dark under 1 atm. carbon monoxide was taken as 100 per cent. The Q_{co} values in the dark under 1 atm. carbon monoxide in Experiments 1, 2 and 3 were 3.3, 10.7 and 12.5, respectively.

As shown in Table II, the partial pressure of carbon monoxide giving half-maximum velocity was about 0.01 atmosphere. The value corresponds to 10^{-5} *M* in the liquid phase, when we assume the solubility of carbon monoxide in the reaction medium was the same as in water. On illumination, the rate of gas uptake was slightly suppressed.

Production of Hydrogen in the Absence of Added Electron Acceptor—As we reported in the previous paper (*13*), the conversion of C^{14}-labeled carbon monoxide into carbon dioxide was observed even when a catalytic amount of viologen dye was employed. It was assumed the oxidation took place according to the following equation;

$$CO+H_2O=H_2+CO_2$$

To confirm the above assumption, the production, of hydrogen was followed mass spectrometrically. The results of the experiment are given in Fig. 4.

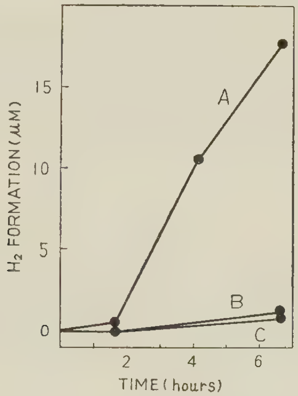

FIG. 4. Production of hydrogen.

Each flask contained 2.0 ml. extracts (boiled extracts in C), 150 μM phosphate buffer, pH 7, 10 μM methyl-viologen and 2 μM sodium formate. Total volume was 3.0 ml. The gas phase was carbon monoxide in A and C, and nitrogen in B. Each flask was vigorously shaken at 30°. Aliquots of the gas samples were drown out at intervals and subjected to mass spectrometry.

DISCUSSION

It is clear from the experimental results mentioned above that we are dealing with an enzyme which activate carbon monoxide.

In the presence of benzyl-viologen as an electron acceptor, the reaction can be described as follows;

$$CO+2Bv^{++}+H_2O=CO_2+2Bv^{+}+2H^{+}$$

where Bv^{++} and Bv^{+} stand for oxidized and reduced forms of benzyl-viologen respectively. When the amount of the electron acceptor was reduced to a catalytic level, the reaction proceeded with the consumption of water as an oxidant. The occurrence of this reaction has been proposed repeatedly by several authors (*6*, *7*, *10*) but has not yet been proved with cell free extracts. It can be inferred that the carbon monoxide activating enzyme and hydrogenase take parts in this reaction together with an electron carrier between them.

As shown in Table I, hydrogenase, formate dehydrogenase and carbon monoxide activating enzyme are different entities, because the rate of heat inactivation of these enzymes are different. The fact that formate dehydrogenase is much more labile against the heat treatment suggests that the formate is not an intermediate in the reaction. The same suggestion was made also in the previous paper according to the results of an experiment in which labeled carbon monoxide was not diluted by the addition of formate.

The effect of illumination on the reaction rate was not big, but the reaction seemed to be suppressed by light. The Michaelis constant, calculated from the results shown in Table II, is $1.4 \times 10^{-5}\,M$ in the dark or $1.7 \times 10^{-5}\,M$ in the light.

D. desulfuricans does not normally utilize carbon monoxide for the growth and the presence of an enzyme activating this substrate was somewhat peculiar. In other microorganisms utilizing carbon monoxide, the same or similar enzyme would take parts in the normal metabolism. The further studies on these subjects are now being undertaken.

SUMMARY

1. An enzyme which catalyzes the oxidation of carbon monoxide into carbon dioxide was obtained in a cell free state from *D. desulfuricans*.

2. The enzyme activity was assayed manometrically by measuring the rate of gas uptake under the atmosphere of carbon monoxide in the presence of benzyl-viologen as an oxidant.

3. The optimum pH range was 7 to 8.

4. The activity was slightly suppressed by illumination.

5. The enzyme was more stable than hydrogenase or formate dehydrogenase against the heat treatment, suggesting that it is a different entity from these enzymes.

6. In the absence of an added oxidant, the enzyme preparation produced hydrogen gas under the atmosphere of carbon monoxide. The phenomenon can be explained assuming the reductive decomposition of water.

The author is indebted to Prof. S. Akabori, Prof. F. Egami, Prof. N. Tamiya and Dr. M. Ishimoto for their enthusiastic interest in and support of this work.

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NOTES

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THE EFFECT OF IRON CONCENTRATIONS ON THE HEME SYNTHESIS BY WHOLE CELL, HEMOLYSATE AND SOLUBLE PREPARATION OF DUCK ERYTHROCYTE

By SEIEI TANAKA, SHIGEKO MORIMOTO, YOSHIKO KAGAWA
SHIGEKI MINAKAMI AND YOSHIMASA YONEYAMA

(From the Department of Physiological Chemistry and Nutrition, Faculty of Medicine, University of Tokyo, Tokyo)

(Received for publication, December 26, 1958)

The synthesis of heme by the hemolysate or the soluble preparation of duck erythrocyte has been reported in the previous papers from the authors' laboratory (*1*, *2*). In this short communication, the authors intended to compare heme synthesis of duck erythrocyte in whole cell suspension, hemolysate and solubilized preparation, taking the concentration of iron needed to elict the half maximum rate of heme formation or the apparent Michaelis constant (K_m) for each system as an index of comparison. The rate of heme synthesis was determined in the presence of various amounts of iron tagged with Fe^{59}. The increase of specific activities after definite intervals of time was considered as the velocity of heme synthesis; the linearity during the experiment had been checked previously. The whole cell suspension in physiological saline solution was incubated without substrates for the addition of either glycine or amino acids mixture seemed to be indifferent to heme synthesis in this system, while to the hemolysate was added glycine to $5 \times 10^{-2}\,M$, in the presence of which maximum heme synthesis was observed. Similarly the solubilized preparation was incubated with $10^{-5}\,M$ protoporphyrin.

Fig. 1-a shows the effect of iron concentration on the iron incorporation into heme in duck erythrocyte by the whole cell suspension. The Lineweaver-Burk plot for this system is given in Fig. 1-b. The K_m obtained from this figure for the whole cell suspension and those for the hemolysate and the solubilized preparation taken from the experiments previously reported (*1*, *2*) were $7.5 \times 10^{-5}\,M$, $9.5 \times 10^{-5}\,M$ and $2.9 \times 10^{-4}\,M$, respectively.

The K_m for the whole cell suspension and the hemolysate were found to be almost identical, whereas that for the soluble system was somewhat greater. The former finding could be explained by the equal potentiality of the iron in- and outside the cell, the penetration of the metal ion into the erythrocyte being only governed by diffusion, as suggested by Wintrobe and his coworkers (*3*). The crossing of iron through the cell membrane might not be the rate determining process of heme synthesis in erythrocyte. The discrepancy

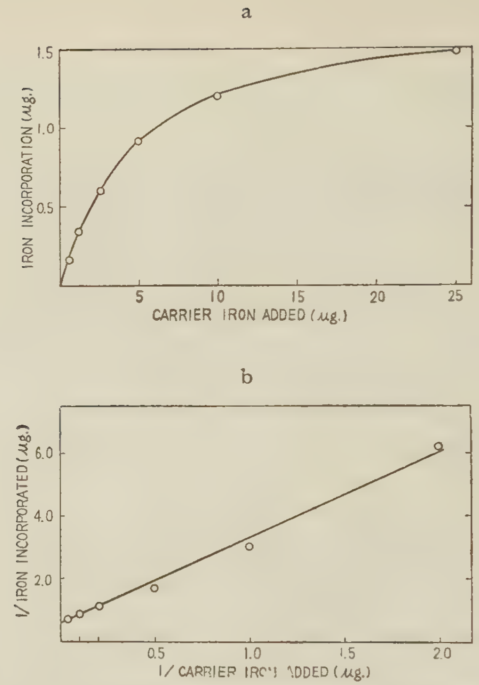

FIG. 1-a. The effect of iron concentration on the iron incorporation into heme in duck erythrocyte. One ml. of Fe^{59}-ferric ammonium citrate solution (1.3×10^5 c.p.m.) of respective iron concentration was added to 10 ml of duck erythrocyte suspension in physiological saline (hematocrit about 35 per cent). The mixture was incubated at 37° for 2 hours.

1-b. The Lineweaver-Burk plot.

between the complex heterogeneous system and simple solubilized system is difficult to explain, since simpler and purer systems have usually smaller K_m. The difference might be due to the existence of cholate or the absence of some factors in the solubilized system.

Thanks are due to Prof. H. Yoshikawa for his kind advice and discussions.

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LETTERS TO THE EDITORS

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MECHANISM OF TRANSPYROPHOSPHORYLATION WITH THIAMINOKINASE

The enzyme thiaminokinase catalyzes the phosphorylation of thiamine by ATP* and its reaction product was identified as TDP, *i.e.* cocarboxylase. The studies of Weil-Walherbe (*1*) and subsequent studies of Leuthardt and Nielsen (*2*) on this reaction suggested a direct pyrophosphate transfer from ATP to thiamine, TMP being less active than thiamine. Although this view has been generally accepted (*3*, *4*) among other possibilities, no conclusive evidence had so far been obtained until the present work was undertaken.

The purpose of the present investigation is to demonstrate the transfer of a pyrophosphate group as a whole from ATP to thiamine with the use of a terminally labeled ATP-P^{32}, and at the same time, to distinguish between two possibilities shown in the following equations:

$$\text{(I)}\quad T+ARPPP^{32} \longrightarrow TPP^{32}+ARP$$
$$\text{(II)}\quad T+ARPPP^{32} \longrightarrow TP^{32}P+ARP$$

However, the main difficulty consisted in the fact that an extremely small amount of TDP was formed and, consequently, the labeled ATP with high specific activities was needed. While the present work was in progress, Khorana *et al.* (*5*) succeeded in demonstrating that pyrophosphate group is transferred by the same mechanism as shown in equation (I), with the enzyme phosphoribose pyrophosphokinase, one of the two enzymes which have been believed to catalyze pyrophosphorylation (*6*). More recently, Greiling (*7*) reported the pyrophosphate transfer with yeast thiaminokinase. He stated that the activity ratio of β to α P atom of TDP, which was synthesized enzymatically from ATP-γ-P^{32}, was 3/1. From this value, the randomization of the labeling pattern of ATP by adenylate kinase or other contaminating enzymes was suspected, although the activity ratio of the ATP used was not specified in his report.

In the present experiments, the yeast thiaminokinase was purified about 100-fold from baker's yeast autolyzate (*8*). With this purified enzyme, the inability of ADP to substitute for ATP and the inactivity of TMP as phosphate acceptor also suggested that a direct pyrophosphorylation of thiamine by ATP is highly probable. ATP-γ-P^{32} was synthesized from labeled carbamyl phosphate by the reverse reaction of carbamyl phosphate synthesizing enzyme (*9*).

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* Abbreviations employed: ATP or ARPPP, adenosine triphosphate; ADP, adenosine diphosphate; AMP or ARP, adenosine monophosphate; TDP or TPP, thiamine diphosphate; TMP, thiamine monophosphate; DEAE-cellulose, diethylaminoethylcellulose; and Tris, tris(hydroxymethyl)aminomethane.

Each P atom of ATP and TDP is distinguished as α, β and γ, and α and β, respectively; AR-P(α)-P(β)-P(γ) and T-P(α)-P(β).

The reaction mixture contained 3 mmoles of Tris buffer, pH 8.6; 150 μ moles of $MnSO_4$; 150 μ moles of thiamine; 15 μ moles of ATP-γ-P^{32} (specific activity 386×10^4 c.p.m. per μmole, γ/β 11.5/1); approximately 6 mg. of purified enzyme; and water to a final volume of 150 ml. The mixture was incubated at 30° for 45 minutes, after which time the reaction was stopped by boiling for 2 minutes at 100°. After centrifugation a suitable aliquot was taken from the supernatant solution and TDP formed was measured manometrically using apocarboxylase prepared from baker's yeast (*8*). About 0.31 μ mole of TDP was synthesized under this condition. The supernatant was adjusted to pH 7.4, diluted about 6-fold, and charged on a Dowex 1 column (chloride form, 4 per cent crosslinked, 1.1×8 cm.). 10 μ moles of nonlabeled TDP were added as carrier.

The column chromatography was performed in accordance with the method of Cohn and Carter (*10*). Thiamine phosphates were separated by successive elution from the column with 0.001 N HCl (for TMP), 0.002 N HCl (for TDP), and 0.01 N HCl (for TTP), respectively. Each peak was identified spectrophotometrically and, after lyophilization, by paper chromatography (*11*). TDP was also identified by its coenzyme activity. The results of the column chromatography showed that the main product of the reaction was TDP. About 70×10^4 c.p.m. of TDP was recovered from the column.

The radioactive TDP was purified by rechromatography on Dowex 1 column (4×0.6 cm.) with the hydrochloric acid system, and its specific activity was determined as 121.4×10^3 c.p.m. per μ mole based on the absorbance at 260 μm. This TDP was hydrolyzed with N hydrochloric acid for 30 minutes at 100°, to split it in a way that differentiates the phosphates of the pyrophosphoryl group. After hydrochloric acid was removed by lyophilization, resulting TMP and inorganic phosphate were separated by column chromatography as above and the specific activity of TMP was found to be 9.7×10^3 c.p.m. per μ mole.

From these results the activity ratio of β to α P atom of TDP produced was calculated to be about 11.5/1, which was equal to that of γ to β P atom of ATP used. The purity of TDP and TMP thus isolated was checked by paper chromatography and both of them were detected as single spots by ultraviolet absorption and thiochrome fluorescence.

The observations described here establish definitely the transfer of pyrophosphoryl group from ATP to thiamine and it seems clear that the mechanism is essentially similar to that of the phosphoribose pyrophosphokinase; thus, as Kornberg (*3*) has already pointed out, the enzyme catalyzes the attack of oxygen in thiamine on the middle phosphorus in ATP with the displacement of AMP by thiamine. According to Westenbrink*, Forsander also reported a paper which came to the same conclusion as the present experiments. Full details of the experiments will be published elsewhere.

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Department of Biochemistry
Faculty of Medicine
University of Tokyo, Tokyo

NORIO SHIMAZONO
YOSHITAKE MANO
RYO TANAKA
YOSHITO KAZIRO

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NUCLEOTIDE SPECIFICITY OF YEAST THIAMINOKINASE: DEPENDENCE ON METAL CONCENTRATION

In the course of the investigation on yeast thiaminokinase, it was found that this enzyme has a broad nucleotide specificity, and it catalyzes the transfer of pyrophosphoryl group to thiamine from GTP*, ITP, UTP, and CTP, besides ATP, although at different rates. Moreover, it was observed that under certain conditions ATP was completely ineffective as phosphate donor, and GTP or ITP was very effective, while under other conditions ATP was much more active than any other NTP. It seemed of interest that the nucleotide specificity depended completely on experimental conditions under which the reaction was carried out, *i.e.* metal concentration and/or pH. This paper describes some evidence for the dependence of nucleotide specificity on the concentration of Mn, and hydrogen ions.

The enzyme was purified about 100-fold from extracts of dried baker's yeast (*1*). The purification process included the following steps: Extraction with 3 volumes of 0.1 *M* Na_2HPO_4 for 3 hours at 37°, heat denaturation of inactive protein at 50° for 10 minutes, acetone precipitation (36–46 per cent), ammonium sulfate fractionation (0.51–0.61 saturation), and DEAE-cellulose column chromatography. The highly purified preparation was free from NDPkinase activity, when assayed by the method of Kurahashi *et al.* (*2*). NTP were the commercial products of the Sigma Chemical Company and the Pabst Laboratories.

The reaction mixture contained: 30 μ moles of Tris-maleate buffer, pH as indicated; $MnSO_4$ as specified; 0.15 μ mole of thiamine; 0.15 μ mole of NTP; and the enzyme to a final volume of 1.5 ml. After incubation for 60 minutes at 30°, the reaction was terminated by heating in a boiling water bath for 2 minutes. A suitable aliquot was taken and assayed for TDP formed. The determination of TDP was made by measuring manometrically carbon dioxide evolution after recombination with apocarboxylase prepared from baker's yeast. 1.5 μ moles of neopyrithiamine was added to eliminate the thiaminokinase activity as a contaminant in the apocarboxylase preparation (*1*).

As may be seen from the data in Fig. 1, pH-activity curve differed markedly with each NTP. ATP was most active in alkaline pH range and the optimum was found to be around pH 8.6–8.8. The pH optima for ITP and UTP were at 6.8 and 8.2, respectively. When GTP was used as

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The following abbreviations were used; NTP, nucleoside triphosphate; ATP, adenosine triphosphate; GTP, guanosine triphosphate; ITP, inosine triphosphate; UTP, uridine triphosphate; CTP, cytidine triphosphate; Tris, tris(hydroxymethyl)aminomethane; and DEAE-cellulose, diethylaminoethyl-cellulose.

phosphate donor, the reaction had a broad pH optimum ranging from 6 to 8. In these experiments the Mn concentration was fixed at $4\times10^{-4}\,M$, and values are expressed as per cent of the optimal activity with each nucleotide. According to the data not presented here, these results should not be interpreted only in terms of pH, because it was noted also that these pH optima depended upon the relative concentration of NTP and activator cations.

A comparison of relative activity with each nucleotide as a function of Mn concentration is given in Fig. 2. In this experiment, pH of the reaction medium was chosen at 8.4 where all the phosphate groups in NTP were known to be in dissociated form. Under these conditions the nucleotide specificity was found to be quite dependent on Mn concentration ; the optimal Mn concentration for GTP was in the neiborhood of $1\times10^{-5}\,M$, where ATP and CTP were practically inactive. At a high concentration of Mn as required for the maximal activity of ATP, very little activity of GTP was obserbed.

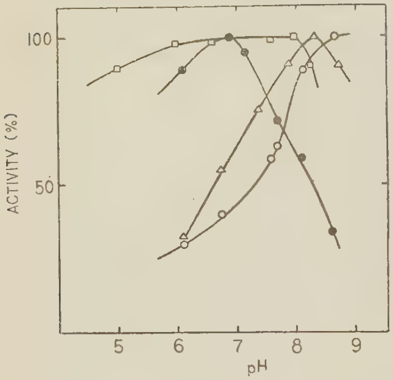

FIG. 1. Activity at fixed metal concentration as a function of pH. —○— ATP, —△— UTP, —●— ITP, —□— GTP.

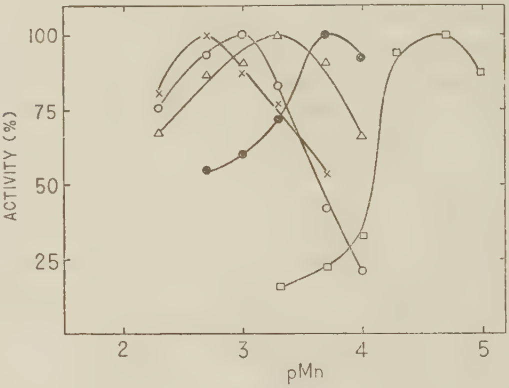

FIG. 2. Dependence of nucleotide specificity on Mn concentration. —×— CTP, —○— ATP, —△— UTP, —●— ITP, —□— GTP.

Thus, each nucleotide had its own pMn optimum and an excess of Mn resulted in an appreciable inhibition of the rate. The values are expressed in terms of relative specific activity in the figure and the activity ratio of each nucleotide at optimal pMn was A : U : G : I : C = 1 : 1 : 0.55 : 0.55 : 0.25.

A question arises, however, as to the possibilities that these results are due to contamination with other enzymes, or that these activities are catalyzed by separate enzymes, each of which is specific for one particular nucleotide. The first possibility was excluded because above results could not be explained by intermediacy of other enzymes and, moreover, NDPkinase activity was not detected in the purified preparation. In order to exclude the second possibility, chromatographic distribution of the enzyme activity was followed with ATP and ITP as phosphate donor. Activity with these phosphate donors was always recovered in the same fraction and with almost a constant ratio during the anion exchange chromatography on DEAE-cellulose column. Furthermore, these activities are by no means additive.

From these results, it seems reasonable to assume that one and the same enzyme is responsible for the reaction and that each NTP would be effective as such, possibly for the reason of broad nucleotide specificity of the enzyme. The metal-NTP complex would also be the true substrate in this reaction as postulated in several other reactions which utilize ATP (*3–6*), and the dissociation constant of this complex would differ with each nucleotide. Metal concentration is of primary importance to the formation of this complex, the formation constant of which is probably influenced by pH of the reaction medium.

It has been known that some enzymes are not absolutely specific for ATP, other NTP being more or less active with the same enzyme preparations. In most of these cases, however, the effectiveness of nucleotide was compared only under the optimal conditions for ATP. Only with ATPase, some papers (*7–9*) have recently appeared which treat the relative effectiveness of NTP under different conditions. The present results certainly stress the necessity of great care in the interpretation of the experiments of nucleotide specificity.

Full details of the experiments will be published elsewhere.

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Department of Biochemistry
Faculty of Medicine
University of Tokyo, Tokyo

YOSHITO KAZIRO
NORIO SHIMAZONO

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課長
小西
岸河 木津
究所に送ります

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